JOURNAL OF AGRICULTURAL RESEARCH

VOL. XXX WASHINGTON, D. C., MAY 15, 1925 No. 10

THE POSSIBILITY OF SEX CONTROL BY ARTIFICIAL INSEMINATION WITH CENTRIFUGED SPERMATOZOA[1]

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INTRODUCTION

The phenomenon of sex is one of the most conspicuous features of living things, both plant and animal. Differentiation of the reproductive organs into two sorts, the one called male and the other female, is almost the universal rule among all but the very simplest of both plants and animals. However, a great variety of arrangements of the male and the female reproductive organs exists in the different groups of the plant and animal kingdoms. Thus the great majority of the higher plants (spermatophytes) and many of the lower forms of animals have both the male and the female reproductive organs in the same individual. On the other hand, some of the higher plants (such as hemp, mulberry, date palm, and many others), and the great majority of the higher animals (including practically all of the vertebrates and arthropods and many of the lower animals), have the male reproductive organs in one individual and the female organs in another.

Sex is popularly considered as a feature of the animal kingdom alone and is scarcely associated with plants. Although this idea of the existence of sex is biologically wrong, it is economically sound because all but a few of the plants of economic importance are monoecious, while all of the mammals, birds, and insects are normally dioecious, and there is frequently a considerable difference in the value of the two sexes of economically important animals.

This article considers the problem of sex control in the light of what is now known about the mechanism of sex determination and gives a detailed report of an experiment carried out at the Wisconsin Agricultural Experiment Station to test the possibility of controlling sex by artificial insemination with centrifuged spermatozoa. This experiment resulted in the discovery of a number of interesting facts, especially in regard to the success and methods of artificial insemination and in regard to the variations in the size of the spermatozoa. It did not succeed in its primary purpose of controlling the sex of the offspring thus produced, but the results of the experiment do throw some light upon the possibility of sex control by such a method and are therefore presented here.

DEFINITIONS

The term "sex determination" is used here to mean the process which determines that any given individual shall be of a definite sex—that is, male, female, or some grade of hermaphrodite. It is an observed fact and must have a mechanism upon which it rests whether or not that mechanism is as yet clearly demonstrated. The term "sex control," on the other hand, is used to mean the modification of the process of sex determination so that the offspring will be of the sex which the breeder desires. The control of sex among the higher nonparthenogenetic animals has not yet been completely demonstrated, and it is quite possible that even a complete knowledge of the mechanism of sex determination will not lead to any successful method of sex control—that is, the problem of sex determination has a solution which may be completely demonstrated at some future time and probably has already been found in the chromosome theory of sex determination or some modification of it, but the problem of finding a practical method of sex control need not necessarily have any solution.

[1] Received for publication July 23, 1924; issued July, 1925. Paper No. 50 from the Department of Genetics, Agricultural Experiment Station, University of Wisconsin, published with the approval of the director of the station.

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REVIEW OF LITERATURE

THEORIES RELATING TO THE DETERMINATION OF SEX

The phenomena of sex play such a large part in human life that they have attracted the attention of investigators and philosophers since the earliest time, and it is not surprising to learn that there have been many theories proposed to account for the determination of sex. Geddes and Thomson (*11*)[3] estimate that there were as many as 500 of these theories at the beginning of the nineteenth century and state that the number has continued to increase. Undoubtedly the increase had not stopped when they made that statement, for the theory which is most widely accepted by scientists at present, the chromosome theory of sex determination, was not suggested by McClung (*28*) until 1902, a year after the revised version of Geddes and Thomson's book was published. The only one of all these theories that has succeeded in winning any considerable amount of both popular belief and experimental support is the chromosome theory of sex determination. The experimental work upon which this paper is based rests upon the chromosome theory as a working hypothesis.

According to that theory the sex of the individual which results from the union of the egg and spermatozoon is determined by whether it contains two X-chromosomes or an X-chromosome and a Y-chromosome. Thus, in the mammals, females contain two X-chromosomes in each of their cells, and each of the egg cells which they produce contains one X-chromosome. Males contain an X-chromosome and a Y-chromosome in each of their cells, and half of the spermatozoa which they produce contain an X-chromosome and the other half of the spermatozoa contain a Y-chromosome. If the egg cell is fertilized by an X-bearing spermatozoon, the resulting individual will contain two X-chromosomes in its cells and will be a female. If the egg cell is fertilized by a Y-bearing spermatozoon the resulting individual will contain an X-chromosome and a Y-chromosome in its cells and will be a male. Sex in birds and in the Lepidoptera is determined in the same way except that the sexes are reversed with respect to chromosome composition. Recent discoveries (*3*, *4*, *5*, *12*, *37*) have made it seem likely that the X-chromosome itself does not determine sex directly, but that its action is due to sex-modifying factors, of which the X-chromosome carries a disproportionately large and unbalanced number but of which the other chromosomes carry a few. This modification of the originally simple chromosome theory of sex determination is of importance chiefly in that it offers an explanation of sex intergrades and has a bearing on the question of the absolute irreversibility of sex.

The proof of the existence of a causal relation between the sex chromosomes and sex determination is so strong that biologists almost universally concede that the sex chromosomes are the mechanism which ordinarily determines sex. However, it may not necessarily follow from this that sex can not be reversed by any unusual combination whatever of the other forces which may have an influence upon sex. Indeed some experiments (*9*, *10*, *18*, *34*, *35*, *36*) have been interpreted as showing that it is occasionally possible to cause an individual to change its sex. There is still much confusion on this point, and until that confusion is cleared up the possibility of complete functional sex reversal can not be disproved, at least among animals like the birds and amphibians in which the two sexes do not differ greatly with regard to the gross anatomy of their organs of reproduction. However, very much of this confusion is due to a failure to distinguish clearly between the primary differentiation into two sexes and the secondary effects produced by the hormone action of the ovary or testis.

Several studies have given results which conform to the chromosome theory of sex determination but indicate that it may be possible to control sex by controlling the chromosome distribution. One of these is the series of studies (*31*, *32*, *33*) of chromosome behavior in groups like the aphids, which reproduce sometimes parthenogenetically and sometimes sexually. In some species the same aphid may produce males or females, or other parthenogenetic females like herself (*38*). Apparently there is something here governing the chromosome behavior, but what it is or to what extent it is "internal" and to what extent it is a result of the environment (and therefore possibly controllable) is not known. Riddle's work (*34*, *35*, *36*) with sex in pigeons seems to demand the same explanation. He claims to have controlled sex in pigeons by the factor of reproductive overwork—that is, the later eggs from females which had been forced to

[3] Reference is made by number (italic) to "Literature cited," p. 912.

lay a very large number of eggs produced a much higher proportion of females than did the earlier ones. Jull (*21*, *22*), working with the domestic fowl, confirmed Riddle's results as to the relation between reproductive overwork and the sex of offspring, but by making use of a sex-linked character in his breeding experiments Jull showed that all the males and females had the chromosome composition normal to their sex. This is added evidence in favor of the chromosome theory of sex determination, and the problem in the work of Riddle and of Jull becomes the discovery of what factor in the condition of reproductive overwork governs the segregation of the sex-determining chromosome into the ovum or the polar body at the reduction division. Hays (*16*) obtained similar results with rabbits.

There have been two notable successful attempts to establish, by selective breeding, strains which produce either an excess of females or an excess of males. The first of these was by Moenkhaus (*30*) working with Dorsophila. He never succeeded in getting a strain which would produce a constant significant excess of males but did succeed almost from the start in getting a strain which averaged about 75 males to 100 females. Warren (*39*), who repeated the same kind of experiment on a different strain of Drosophila without success, showed that the results of Moenkhaus harmonized perfectly with the idea that he was dealing with a strain which carried a sex-linked lethal factor, and since more than 20 such sex-linked lethal factors have already been found in *Drosophila melanogaster* this case seems to support rather than to disprove the chromosome theory of sex determination. The second successful attempt to establish excess-male and excess-female lines by selective breeding was that made by King (*23*), using albino rats. King's results also conform fairly well to the hypothesis that her initial stock contained lethal factors. The results in the low male line fit the hypothesis that she was dealing with a strain containing a sex-linked lethal factor very well, except for the fact that the inbred females when bred to stock males produced a somewhat larger proportion of males than when bred to full brothers. The results in the high male line fit even better the hypothesis that she was dealing with a strain containing a sex-linked factor which, when homozygous, is lethal for females but has no effect upon males. (A similar factor has been reported (*2*) in Drosophila.)

RELATION OF THE CHROMOSOME THEORY OF SEX DETERMINATION TO THE PROBLEM OF SEX CONTROL

The existence of complete functional sex reversal is still in question even when considering birds and amphibians. Moreover, those cases which have been reported as proving such sex reversal in birds (*9*, *10*, *35*, *36*) do not offer proof as to how such sex reversal might be controlled by the breeder. Furthermore, Lillie's work (*24*, *25*, *26*) on the freemartin has made it appear that complete sex reversal among the mammals is still more unlikely. Aside from the possibility of sex reversal, the problem of sex control may be approached from two sides—either with the idea of destroying the fertilized eggs which are of the undesired sex, or with the idea of preventing the formation of such fertilized eggs.

The work of Riddle (*34*), Jull (*22*), and Hays (*16*) may furnish a clue to some method of sex control, but as yet the underlying principles governing the partial success which they obtained in the control of sex have not been clearly demonstrated and have not been shown to be as completely susceptible to human control as would be desirable in a practical method of sex control. Killing or rendering impotent the spermatozoa which would produce the undesired sex seems to be a simpler method of attack, because treatment of the spermatozoa with a poisonous chemical agent could be in vitro, and the female could afterward be fertilized by artificial insemination.

Separation of male-producing and female-producing spermatozoa by physical means seems more possible than by chemical means. The physical differences upon which separation seems most possible are differences in motility and differences in size. A difference in motility might itself be due to a difference in size which would result in the smaller spermatozoa offering less resistance to forward motion through the liquid by which they are surrounded. This is a possible explanation for the normal excess of males born among most species of mammals. In order to take advantage of differences in motility it would be necessary to keep the spermatozoa oriented in one direction. Possibly this might be done by means of an electric current, but, so far as the author knows, this has not been tried. A physical separation of the spermatozoa upon the basis of their size difference hardly seems possible by any filtering or straining method because of their smallness and delicacy. However, separation of the two sizes

by means of centrifugal force exerted upon them while they are immersed in a liquid of very nearly the same density as themselves is possible theoretically, and since some preliminary experiments along that line had indicated that success might actually be obtained in this way the experiments which are reported in this paper were designed to test that point.

DIMORPHISM IN SIZE OF SPERMATOZOA

The existence of two distinct sizes of spermatozoa in many animals is to be expected, particularly in those in which the Y-chromosome is absent or very much smaller than the X-chromosome. The head of the spermatozoon is composed almost entirely of chromatin, and therefore the spermatozoa which receive an X-chromosome should have larger heads than those which receive the Y-chromosome except in cases where the Y-chromosome is as large as or larger than the X-chromosome. This has actually been found in a number of species of vertebrates and insects (*43*, *44*, *45*, *46*, *47*). Considering that the size of the spermatozoon head depends upon three dimensions and possibly may vary in any one or all of these dimensions more or less independently, it is remarkable that the results obtained show rather uniformly two size groups in nearly equal numbers when only the head lengths were measured. However, each species has a rather characteristic shape for its spermatozoon heads and the length of the head may be a fairly reliable index to its total size. Two classes of spermatozoa have been found, the differences between their mean head lengths approximating very closely in most of the species the theoretical differences calculated from drawings of the chromosomes as seen during maturation divisions of the germ cells.

EXPERIMENTAL PROCEDURE

FACTORS INVOLVED IN THE PROBLEM

In undertaking experimental work involving the separation of spermatozoa by centrifugal force, several factors had to be considered. If there is no difference in the density of the two sizes of spermatozoa the larger ones will offer less resistance than the smaller ones, per unit of weight, to locomotion through a surrounding liquid, whether the specific gravity of the liquid be greater or less than their own, because the volumes of the larger ones (and therefore their weights, upon which depend the forces tending to move them through the liquid) are proportionately greater per unit of surface or cross section (upon which depends the resistance which they offer to locomotion through the liquid). If the specific gravity of the liquid were very much more or very much less than that of the spermatozoa themselves, a very little centrifuging would cause both classes of spermatozoa to go either to the inside or to the outside of the centrifuge tube together, but if the specific gravity of the liquid were very nearly that of the spermatozoa their progress during the centrifuging would be very much slower, and if the right amount of centrifugal force were used for the right length of time a higher percentage of the large than of the small spermatozoa would reach the inside or the outside of the centrifuge tube, according to whether the specific gravity of the liquid were more or less than that of the spermatozoa. If, however, the two sizes of spermatozoa differ in specific gravity, a thing for which there is no proof and which there are no a priori reasons to expect, but which might nevertheless be true because the chromatin of the sex chromosome might be of a different specific gravity from that of the other chromosomes, the problem becomes somewhat different. If, in this case, the specific gravity of the liquid could be made intermediate to that of the two classes of spermatozoa, it would be possible to effect a more complete separation than under any other conditions, for prolonged centrifuging should result in one class going to the inside and the other to the outside of the centrifuge tube. If the larger ones have the greater specific gravity, they would move through the liquid still more rapidly than if there were no difference in specific gravity, since in addition to having less resistance per unit volume they would also have a greater weight per unit volume than the smaller ones. If the smaller ones have the greater specific gravity, this would counterbalance their greater resistance per unit volume, and the only means of separating them by centrifugal force would be to adjust the specific gravity of the liquid to a value intermediate to that of the large and of the small spermatozoa.

Thus, there are at least six important factors which influence the success of the centrifugal method of separation of the male-producing from the female-producing spermatozoa. The first is the difference in size of the two classes, a factor which is not under control at

all and which is of cardinal importance. The second factor of success is the difference, if any, in the specific gravity of the two classes of spermatozoa, which also is not under control and moreover is not readily ascertainable. The third and fourth factors are the specific gravity and viscosity of the surrounding liquid. These could be controlled within limits by the addition of water or of certain solutes, provided this was not carried so far as to injure the spermatozoa. A more serious difficulty in the way of controlling the specific gravity and viscosity of the liquid lies in the fact that the liquid as recovered from the vagina and uterus of the female by a catheter varies so greatly in those properties that it would be necessary to determine them anew for every sample. The fifth and sixth factors are the amount of centrifugal force used and the length of time for which it is applied. These are quite within the control of the experimenter, but he has no way to determine the correct time or the correct force to use except by trial. Moreover he can only know whether he has succeeded by a microscopic examination (measurement) of the spermatozoa in samples from the inside and from the outside of the centrifuge, which would require a good many hours of continuous work and practically could not be carried out until the spermatozoa were too old to be viable, so that no advantage could be taken of the knowledge. It will readily be seen that the difficulties in the way of finding and maintaining the correct technique are very many and very serious, but nevertheless they did not seem insuperable.[4]

MATERIAL

The rabbits used were of mixed stock, small or medium in size, derived largely from such varieties as the Black Dutch, New Zealand Red, Himalayan, and ordinary albinos and other colors of mixed ancestry. The advantages of the rabbit as material for work of this sort are that it is of very convenient size for operation and for artificial insemination; that the period of gestation is very short and the size of litter is large, thus permitting the production of large numbers in a very short time because the young can be killed at birth if their sex is the only point on which information is wanted; that they breed all the year (to some extent, although more readily at some seasons); and finally, that they are rather cheaply maintained, and since they are mammals it is reasonable to suppose that principles learned from them would apply also to other domesticated mammals or to man. The disadvantages of using the rabbit are that the females are quite subject to infection of the uterus and Fallopian tubes by pus-forming bacteria and the difference in size between the X-chromosome and Y-chromosome is not very great (*1*).

The material from swine was obtained from three different Poland-China boars from the University of Wisconsin herd. Two of these were mature and one was about 10 months old. The two mature ones were castrated and the testicles, in the one case immediately and in the other case after immersion for about three hours in water at about 25° to 35° C., were opened with a knife and a milky fluid which oozed out from the many cut ends of the epididymis was collected by means of a catheter and a rubber bulb. The young boar was allowed to copulate with a sow and the fluid which could be recovered from the sow immediately afterward with a catheter and rubber bulb was used for the microscopic studies. Swine are too slow breeding and too expensive to maintain for the carrying out of the actual breeding experiment unless there should be unusually good reasons to expect success with them. The chromosome composition of swine as reported by Wodsedalek (*43*) is such as to give more reason to expect success in the centrifugal separation of the two classes of spermatozoa in this animal than in most, or perhaps than in any other which has yet been studied. It should be added that Hance (*14*) could not confirm Wodsedalek's results. According to Wodsedalek, the male-producing spermatoza contain 8 and the female-producing spermatozoa 10 chromosomes, and, as nearly as can be judged from his drawings, the 2 chromosomes which together behave as a single X-chromosome constitute 20 to 30 per cent of the volume of the chromatin in the spermatozoa which contain 10 chromosomes—that is, the volume of the male-producing spermatozoon might be expected to be 70 to 80 per

[4] The work was done in the laboratories of the department of genetics at the University of Wisconsin. It was originally planned by and was continually under the direction of L. J. Cole. Some preliminary work by Sarah V. H. Jones and L. J. Bachhuber had indicated that mechanical separation of the male-producing and female-producing spermatozoa might be possible by means of centrifugal force, and therefore that sex control would be possible by artificially inseminating the females with only the desired kind of spermatozoa. On the strength of this preliminary showing a detailed test of this possibility was made, using rabbits for the experimental animals but also making a few tests on semen from swine. The microscopic work was done with the advice and assistance of M. F. Guyer of the zoology department.

cent of that of the female-producing spermatozoon. His measurements of head lengths of mature spermatozoa also show a very distinct dimorphism, the smaller mode being 82.5 per cent of that of the larger one, which is a very close check with the calculated difference when it is considered that he was measuring only one dimension, whereas volume would depend upon three dimensions. The main facts as to the suitability of the animals used are contained in the statement that rabbits are excellent material in every respect but one—their chromosome composition—and that swine are excellent material chiefly in that one respect alone.

PLAN OF EXPERIMENT

The plan of the breeding experiment was to secure normal rabbit spermatoza immersed in the liquid which naturally surrounds them in a normal copulation, then to subject them to centrifugal treatment, then to inject various portions of the centrifuged liquid into the vagina or uterus of the female rabbits which were in heat, and finally to observe the sexes of the offspring resulting from these different kinds of artificial insemination.

FEMALE BEHAVIOR

It was necessary to know whether a female was in heat, and since there is no more certain sign of oestrus in the doe rabbit than her willingness to copulate with the buck, a number of sterile males were provided to act as teasers or test males to determine whether a female was in heat and yet to avoid having her become pregnant to a natural service. There are other indications of oestrus, more or less reliable, such as the degree of congestion or swelling of the vulva, mounting the other females, etc., but none of these indications was completely reliable for all the female rabbits. Each female had a type of behavior, when in heat, to which she adhered rather consistently, and it was possible after becoming acquainted with her individual record to predict rather accurately whether she was in heat without actually placing her with the male. However, the method of placing the females with the test males was always relied upon as being more accurate and almost as convenient as any other. If the doe was in heat, she usually accepted the buck almost immediately when placed with him, and if she did not accept him in the first minute or two there was very little probability of her accepting him at all, no matter how long he was allowed to tease her; but there were exceptions to this, and very rarely a doe that had refused one buck for as much as 10 or 15 minutes would accept another almost immediately when placed with him.

TEST MALES

The test males were made sterile by the operation of vasectomy, which is very easily performed on the rabbit. The test males varied in their behavior and in the ardency of their sexual desire, but so did the normal males, and although no method of making a measurement of this feature was devised, close observation leads to the conviction that the males which were not vasectomized until they were sexually mature were no less ardent and no less active sexually than the perfectly normal males. Whether the males vasectomized before they were sexually mature were affected by the operation at all remains somewhat uncertain. Some of them did develop normally and became quite ardent, but there were others which always remained rather weakly and never became ardent enough to be satisfactory test males. However, this was also true of some of the untreated males. There was nothing in this work to prove or even to indicate very strongly that the operation of vasectomy produces any psychic changes (*40*).

RECOVERY OF SEMEN

Ejaculation can not be induced in the rabbit by artificial stimulation and the use of a vaginal sponge or a condom is not practical (*27*), so the seminal fluid was recovered by allowing a buck to serve a female normally and then recovering as much fluid as could be obtained from vagina and uterus by means of a catheter and rubber bulb. This method seems to be the only practical one for an experiment of this kind on rabbits, and moreover it is simple and easily operated and the amount of fluid which can be recovered is large; but it has the disadvantage that any secretions or cellular residues or bacteria within the vagina are also drawn into the catheter and any effect which they may produce upon the spermatozoa can not be separated out. Moreover, an infection is readily spread from female to female by this method, since the fluid can not be sterilized without killing the sperma-

tozoa. Also it is impossible to be certain that all the spermatozoa ejaculated by the buck have been removed and therefore the female from which the seminal fluid has been recovered can not be used for an artificial insemination with a centrifuged portion of that fluid. In fact, the records show that in this experiment the females from which as much fluid as possible was recovered were more likely to become pregnant than were those which were inseminated artificially. This furnished an excellent control for the insemination experiments, but it made necessary the use of a much larger number of females. An attempt was made to avoid this difficulty by using for recovering the semen females upon which the operation of salpingectomy had been performed. Since they could not become pregnant, they could be used very frequently and a small number of them would suffice for recovering enough seminal fluid to inseminate artificially a very large number of normal females. However, in practice this did not work out so well because these females did not come in heat any oftener than normal females, that is at intervals of 12 to 15 days, and a large number of them would have been necessary if one were to have been available every time a normal female was to be inseminated. Moreover, after several months the females upon which salpingectomy had been performed became very fat and almost entirely ceased to come in heat, and, since a number of the normal females would accept service even when they were pregnant, the use of operated females was discontinued. Only three such females were used in this experiment, and the conclusions derived from their behavior are of little value by themselves, since individuality is such a large factor in determining behavior, but the conclusions reached agree very well with those reached by Lloyd-Jones and Hays (*27*) from more extensive observations.

Ordinarily all the females which had not been bred within the preceding 10 days were tested each morning or every alternate morning to find out whether they were in heat. When two or more were found in heat the same morning, a buck was allowed to serve one of them naturally as many times as he would in about 15 minutes, which was usually twice, but sometimes three times or more, and more rarely only once. Then the female was removed and all the fluid that could be recovered from her vagina and uterus by two or three successive insertions of the catheter was obtained. If the amount of fluid was large enough to be handled easily, the female was returned to her hutch and was not tested again for 10 days, but if the amount was too small the catheter was filled with warm Ringer's solution which was injected into the uterus and allowed to remain 2 or 3 minutes. Then the catheter was again thrust in and all the liquid possible was withdrawn. In this way more semen was recovered, diluted to a somewhat uncertain extent, so that it could be handled and centrifuged very conveniently.

The catheters[5] used were made from pieces of glass tubing with an outside diameter of 5 to 6 mm., with a slight bend near one end and a bulbar expansion near the other. The total length was 18 cm., and the part which could be thrust into the vagina and uterus of the female was about 12 to 12.5 cm. long. The uterus of the rabbit does not have a very definite cervix or os, and the body of the uterus is short and small while the horns are relatively long. Because of this, the catheter was thrust not only into the vagina but also into the uterus and usually even a considerable distance into one of the horns of the uterus when withdrawing liquid.

CHARACTER AND TREATMENT OF THE FLUID

The fluid recovered consisted of a combination of semen and vesicular and prostatic secretions from the male and the vaginal and uterine secretions from the female. It was usually opaque and of a pale milky color; sometimes it was of a dense yellowish or milky white color, and sometimes it was almost transparent. If recovered from a female within two or three days after parturition it usually had a reddish or brownish tinge and evidently contained some blood. Sometimes urine would be contained in the liquid. This would usually give it a rather transparent yellow color and the spermatozoa would be found to be nearly or quite motionless. Often there were very many round motionless cells about the structure of which very little could be made out. These were often associated with cases of known pus infection in the uterus of the female from which the fluid was drawn, but whether this relation was significant

[5] This catheter or pipette, first devised in this form by Bachhuber, has been used and figured by Guyer in his "Studies on Cytolysins" (*13, p. 208*).

is not known. Often there were transparent gelatinous masses as much as 2 or 3 mm. in diameter. No attempt was made at a chemical analysis of the liquid or to determine its physical properties except such as were visible. The liquid was examined microscopically as soon as recovered. If no active spermatozoa were found, the liquid was discarded, but if there was a fair number of active spermatozoa, the liquid was used.

Sometimes the liquid was centrifuged untreated, but usually in order to get a convenient volume it was diluted to some degree with Ringer's solution. The only noticeable effect of dilution was that it resulted in a more distinct separation of the spermatozoa and cells from the liquid upon centrifuging and fewer of them were found in the inside of the centrifuge tube. Presumably, therefore, it decreased the specific gravity or the viscosity of the fluid.

The centrifuging was done in the earlier experiments with a Bausch & Lomb hand centrifuge with which it was possible to govern the centrifugal force by regulating the speed at which the operator turned the handle. The speed ordinarily used was about 1,600 r. p. m. The later experiments were carried out with a Bausch & Lomb electric centrifuge which had a lowest speed of about 1,600 r. p. m. and a second speed of about 2,000 r. p. m. The liquid was placed in the centrifuge in glass tubes 12.5 cm. long with an inside diameter of about 6.5 mm., and therefore a capacity of about 4.15 c. c. They were so arranged that when revolving the extreme bottoms of the tubes were about 30 cm. apart and the tops were about 5 cm. apart. Therefore, the amount of centrifugal force applied varied in the different regions of the tube. The centrifuge tube containing the liquid was kept in a water bath at a temperature of 35° to 37° C. until it was placed in the centrifuge which was at room temperature. This naturally varied somewhat at different seasons. After the centrifuging, the tubes were again placed in the warm-water bath before injection. The length of time during which the tubes were centrifuged varied from 2 to 10 minutes. When prolonged much beyond 10 minutes, the spermatozoa were so nearly all thrown to the bottom of the tube that it seemed useless to try insemination with the liquid from the top.

INSEMINATION

The liquid was injected into the uterus of the females with another catheter which was so long that nearly all the liquid must have been deposited in the upper end of the uterus or in one of the horns. Some females were inseminated when not in heat and others showed unmistakable signs of heat but had not actually copulated with the test males. This was done to test whether copulation is necessary to ovulation in the rabbit as maintained by Marshall (*29*). Some were inseminated with the untreated liquid just as it was recovered. Others were inseminated with liquid which, aside from dilution, had received no treatment except that it had been allowed to remain in test tubes at low temperature for periods of from 1 to 48 hours. However, the majority were inseminated with centrifuged material and these were grouped in two classes according to the part of the centrifuge tube from which the material was taken. The top one-third of the liquid in the tube, that which had been on the inside while the centrifuge was running, was carefully drawn off with the catheter, and females inseminated with this portion were recorded as "inside insemination." The middle one-third of the fluid was discarded entirely except that occasionally microscopic slides were made from it. The bottom or outer one-third, which also contained the sediment, was stirred up and injected into a single female. Such inseminations were recorded as "outside inseminations."

ISOLATION OF FEMALES

Females used for breeding were kept in individual hutches from which they were removed only for testing whether they were in heat. All females when bred in any manner were left in their hutches for 10 days before being again tested.

CHECKING THE SEX OF THE OFFSPRING

The sex of the living rabbit can not often be identified before it is 3 weeks old and sometimes not until it is 8 weeks old. To make possible the production of a larger number of offspring from a given number of females, the young were often killed immediately after birth and the mother bred again, as that is one of the surest times to

induce pregnancy. The young killed in this way, together with those which died in the first few weeks, make up about half of the total number produced, and their sex was identified solely by autopsy. All young or partially grown rabbits which died were also autopsied to verify the records of their sex as determined from external examination. The ovary and testis have very distinctive shapes in the young rabbits even when just born. This is due largely to the distinct firm union of the epididymis to the testis and the small bulb where the epididymis curves at the anterior end of the testis as contrasted to the loose attachment of the Fallopian tube to the ovary. The positions of the ovary and testis are not so distinctive, for the testis is usually just starting to descend when the rabbit is born; but it descends in a very few days and, moreover, the *gubernaculum testis* is so prominent even at birth that it usually would serve for identification. Taken in connection with the shape and position of the testis, there is no room for doubt as to the sex of a young rabbit unless it has been partly eaten by the mother or has been dead long enough to have started to decompose. The female rabbit quite frequently eats her newborn young, and these, together with others about which the records are not quite clear, constitute the group of "unknown sex" in these records.

MICROSCOPIC WORK

To parallel the breeding work with the rabbits and serve as a control by determining the effect of centrifuging upon the measurements of spermatozoa, smears were made of the untreated fluid just as it was recovered from the uterus or after dilution. Again after centrifuging there were smears made both of the "inside" and "outside" material and sometimes of material from intermediate positions. The material was placed upon a clean slide or upon one which had been thinly coated with fresh albumen and was either heated first to fasten it to the slide and then was fixed in Bouin's fluid or was fixed in Bouin's fluid direct without any heating. As good results were secured without as with the heating, except that when the material had been much diluted there was sometimes not enough of its own protein to fasten it to the slide without heating and it washed off while being fixed in the Bouin's fluid. The smears were stained in Heidenhain's iron haematoxylin or in Delafield's haematoxylin, with eosin as a counter stain.

The head lengths of a large number of spermatozoa were measured on a number of these slides, but since this work necessarily requires a great deal of time only a small fraction of the whole number of slides made could be measured. The first measurements were made with a Spencer micrometer eyepiece, but all the later measurements were of drawings made with a camera lucida, as this made it unnecessary to touch the microscope tube at all during measurement and thus removed a possible slight error caused by jarring the tube when touching the micrometer screw. However, when the accuracy of both methods was tested by measuring 100 spermatozoa twice the micrometer method was found to be slightly more accurate. The coefficient of correlation between first and second measurements was $+0.671 \pm 0.037$ for the camera lucida method and $+0.761 \pm 0.028$ when the ocular micrometer was used. Seven hundred spermatozoa were measured on each of four normal slides, two containing the outside centrifuged and one the inside centrifuged liquid. The "inside" slide, one of the "outside" ones, and one of the normal ones were all made from the same sample of recovered liquid.

Microscopic work was also done on swine spermatoza to determine the effect of centrifuging. Smears were made, in the same way as described for the rabbit, of the normal material and also of the material as taken from different positions in the centrifuge tube immediately after centrifuging. Measurements were made entirely by the camera lucida method of 700 spermatozoa on each of three normal slides, three "inside" slides and one "outside" slide.

VITALITY OF SPERMATOZOA

A number of observations were made upon the effect of heat and cold upon the length of time spermatozoa remained motile. A bull was killed and the testicle was immediately removed and placed in a refrigerator and examined daily afterward by making a fresh cut in the epididymis and looking for motile spermatozoa in the liquid which oozed out. A few motile spermatozoa were still found on the eighth day, but through an oversight all the ice was allowed to melt away that afternoon and on the ninth day the odor of putrefaction was quite marked and no motile spermatozoa could be found. In the course of the work on the swine and rabbit spermatozoa the liquids were often kept in a test tube at temperatures varying

from 0° to 20° C. until all motility disappeared or until an odor of putrefaction was noticeable. In the case of the lower temperature some motility was observed as long as 60 hours after ejaculation in some samples, and almost always as long as 48 hours. With temperature as high as 18° to 20° all motility disappeared sooner, sometimes in less than 24 hours.

Temperature was not recorded except at the time of observation, and temperature control was not accurate, so that the only conclusion which can be drawn is the general one that spermatozoa when kept in a liquid in vitro retain motility much longer at lower than at higher temperatures, and therefore that the checking of their activity is due to bacterial growth in the medium or the accumulation of the by-products of their own activity or the exhaustion of their own supply of energy due to this greater activity, rather than to the mere lapse of time. Possibly they would remain active much longer inside the uterus where the toxic products of their own or of bacterial activity would diffuse away to a considerable extent. Not all the spermatozoa in the same sample remained active for the same length of time, but usually a great many were inactive at the end of 24 hours, even though some remained active for more than 48 hours. Whether motility is a sufficient index of fertilizing ability is not revealed by these data. There is only the fact that two litters were produced after insemination with liquid which had been kept for 24 hours, in the one case at 10° to 18° C. and in the other at 13° to 17°.

DISCUSSION OF RESULTS

SEX OF ALL RABBITS PRODUCED

Table I presents the sex of all the rabbits which have been produced in the flock of the genetics department of the University of Wisconsin in the last 10 years. The classes marked "alcoholized" or "lead" are from former experiments (*6*, *7*), the plan of which was to mate albino females to two males on the same day, one of the males being normal and the other in a chronic state of lead or alcohol poisoning. One of the males was an albino and the other was homozygous for the pigment-producing factor and therefore the paternity of the young produced could be known. The individuals in the class marked, "dam had not copulated" are counted again in their appropriate insemination class, but are only counted once in the grand total. Also the individuals of the "fluid washed out" class are counted again in the "fluid withdrawn" class, but are only counted once in the grand total. The next to the last column in Table I gives the probable error to be expected on the basis of exact equality in the numbers of each sex. The last column in Table I shows for each method of breeding just what proportion the deviation from exact equality bears to its probable error and therefore whether these deviations are likely to be the result of anything but chance.

In this and the following tables the probable errors and the deviations are calculated upon the basis of an expectation of exact equality in the sex ratios. As far as the chromosome theory of sex goes, equality is to be expected, but, as

TABLE I.—*Sex of rabbits produced in flock of the genetics department, University of Wisconsin, during 10 years*

Type of litter		Sex			Probable error 1:1	Deviation—probable error
		Males	Females	Unknown		
Sire alcoholized		6	12	7	1.43	2.10
Lead	Sired by poisoned buck	102	93	20	4.71	.96
	Sired by normal buck	74	63	* 32	3.95	1.39
Artificial insemination	Dam had not copulated	12	10	3	1.6	.63
	Fluid untreated	11	17	7	1.8	1.68
	Fluid cooled	18	23	6	2.16	1.11
	Centrifuged, "inside"	64	64	3	3.82	----------
	Centrifuged, "outside"	103	97	14	4.77	.63
Natural service	Fluid withdrawn	170	205	28	6.53	2.68
	Fluid washed out	18	27	1	2.26	1.99
	No treatment	421	394	174	9.63	1.40
Grand total		969	968	291	14.84	.03

* Some of these may belong in the row above.

already mentioned, observation has apparently shown that there are slight but consistent deviations which vary for different species (*33*). Among most mammals it seems that there is a slight excess of males, the normal sex ratio being usually somewhere between 103 and 110 males per 100 females. This may very easily be due to some difference in the motility or viability of the two classes of spermatozoa. The presumption is that in the rabbit also there will normally be a slight excess of males, and the data from this flock for the litters produced by a natural service without any treatment afterward support that assumption, for there were 421 males and 394 females, a ratio of 106.9 ♂ ♂ : 100 ♀ ♀.

However, no report of large numbers has been found for the rabbit. The numbers available are too small for calculations to be based upon ratios drawn from them for a standard, and therefore the calculations in these tables are based upon exact equality as a rigid standard to be given up only when enough data are available to establish definitely how far the normal sex ratio in the rabbit does deviate from equality. The probability that the normal sex ratio is somewhere between 105 and 108 males per 100 females should be kept in mind, however, in considering the results given in these tables.

The common method of stating sex ratios in terms of the number of males per 100 females is open to criticism in that a change in the number of males produced is not given equal numerical value with an equal change in the number of females produced. Thus in a population where the sexes are equal, if a change were made which reduced by one-fourth the number of males born the sex ratio would be given at 75 ♂ ♂ : 100 ♀ ♀, whereas in the same population if the change had reduced the number of females produced by one-fourth the sex ratio would have been stated as 133⅓ ♂ ♂ : 100 ♀ ♀. In the one case the ratio would be described as 25 points below the normal and in the other as 33⅓ points above the normal, although the magnitude of the changes had been the same fundamentally, but took place in different sexes in the two cases. This seems to be a minor point, but it tends to obscure what may be a very vital relation. For example, in her work on selection for abnormal sex ratios in the rat, King (*23*) states that in the high male line after the seventh generation the sex ratio was 122.3 ♂ ♂ : 100 ♀ ♀, while in the low male line for the same period it was 81.8 ♂ ♂ : 100 ♀ ♀. Now it is not readily apparent without calculation that a ratio of 81.8 ♂ ♂ : 100 ♀ ♀ is the same as a ratio of 100 ♂ ♂ : 122.25 ♀ ♀, or almost exactly the same as the ratio in the high male line except that the sexes are reversed. If these ratios had been expressed in percentages, that fact would have been apparent at once and, taken in connection with the fact that selection appeared on the whole to have very little effect after the twelfth generation, would have brought home a conclusion not emphasized in that paper—namely, that in the albino rat selection can alter the sex ratio in either direction with equal ease but can only carry it the same distance on either side of equality. That fits in very well with the hypothesis that the results which she secured were due to sex-linked lethal factors or factors whose lethal action was confined to one sex, and suggests that the ratio which she adopts as a normal, 105 ♂ ♂ : 100 ♀ ♀, is really the result of some such factor as a difference in motility of the two types of spermatozoa. There seems to be no good reason for expressing sex ratios in terms of the number of males per 100 females except that it is customary and magnifies the differences between ratios.

The attempt to develop a practical method of sex control by means used in these experiments was not successful. In Table I, where the different methods of breeding are compared, the only sex ratio which deviates far enough from equality and concerns numbers enough to approach statistical significance is that of the young produced by a natural service where all the fluid possible was withdrawn with a catheter. If the normal sex ratio for the rabbit be taken as 106.9 ♂ ♂ : 100 ♀ ♀, as it was found to be with the litters produced in the normal way in this flock, then the deviation of this class from expectation becomes more than 3.64 times the probable error, and, looking at it from a purely statistical standpoint, it may be said that the deviation is probably significant. However, when it is asked what factor has changed the sex ratio and how it has operated to produce this result, the answer is not apparent. The only readily apparent change which has been made in this type of breeding is a reduction in the number of spermatozoa which have been left in the uterus and vagina, but just how this could influence the sex ratio is not apparent. A reduction in the number of spermatozoa present might cause an already abnormal sex ratio to approach nearer equality in a way analogous to the differential

growth rates of pollen tubes as shown by Correns (*8*), but that a reduction should cause the sex ratio to deviate much more on the other side of equality is hardly explainable upon that basis.

One other effect which the withdrawal of the seminal fluid with a catheter might have had is to carry parts of the fluid much farther up into the uterus than a natural service would have done, but even if this were done to a considerable extent there does not seem to be any reason to think that more of the female-producing than male-producing spermatozoa would be carried up into the uterus nearer to the unfertilized ova. Furthermore, if it were only a matter of pushing the spermatozoa far up into the uterus, all the litters produced by artificial insemination should show a very high proportion of females, whereas actually there was only a bare majority of females. Since there is apparently no physical basis for this abnormal sex ratio, judgment must be reserved as to its real significance until there are more data available. However, it is certain that none of the other methods of breeding has produced a significant change in the sex ratio, for if, instead of equality the ratio of 106.9 males be taken as normal, the deviations from expectation become in general even less than they are when based upon equality.

SEX OF OFFSPRING IN RELATION TO LENGTH OF GESTATION PERIOD

That there is no relation between the sex of the offspring and the length of the gestation period is shown by Table II. Here the nearest whole number of days between the hour of breeding and the hour of finding the litter is given. Since many of the litters may have been as much as 12 hours old when they were found, it is apparent that while some of them may belong in a period one day shorter than that in which they are placed, none of them belong in a longer period.

That the length of the gestation period would vary with the sex ratio of the litter is hardly to be expected, since nearly every litter contains both males and females, and even if the males require a longer or a shorter time to complete their development still the litter must all be born at the same time and only the exceptional cases where the litter is all of the same sex might be expected to show this relation. Since the extreme gestation periods as shown in Table II do not give extreme ratios and since the average gestation

TABLE II.—*Sex of offspring and length of gestation period*

Length of gestation period	Sex of offspring			Deviation—probable error
	Males	Females	Unknown	
Days				
Unknown	179	144	71	2.89
26	1	3		
28	10	6	1	
29	15	13	1	
30	114	134	44	1.88
31	175	198	29	1.77
32	104	101	13	.31
33	16	17	2	
34	11	8	2	
35	2	4	1	
39		3		

period of the female offspring was less than 0.02 days in excess of that of the males, it may be concluded that in the rabbit the sex of the offspring bears no relation to the length of the gestation period. There may, however, be some such relation in uniparous animals where an embryo of one sex during its entire course of development is free from possible influences from embryos of the other sex in the uterus beside it, and it is a fairly common belief among breeders of horses and cattle that there is such a relation in those animals.

SEX RATIOS OF PROGENIES OF INDIVIDUAL MALES AND FEMALES

That such deviations from a normal sex ratio as may exist are not due to the progeny of a single male which happen to be grouped together is shown by Table III, which gives the sexes of the progeny of all males which have as many as 50 progeny whose sex is known.

TABLE III.—*Sex of progeny of males*

Sire	Sex of offspring			Deviation—probable error
	Males	Females	Unknown	
274.3	32	30	18	0.38
301.3	45	50	6	.76
316.1	31	47	29	2.68
411.1	53	46	11	1.04
397.1	60	70	8	1.30
440.1	39	36	3	.51
391.1	37	46	7	1.46
515.4	22	28	8	1.26
534.1	47	38	8	1.48
20.2	28	23	7	1.04
20.2 and 21.4 (Lead experiments)	73	70	3	.37
26.8 and 20.2 (Lead experiments)	69	69	38	

Table IV presents the corresponding facts for all females which have 25 or more offspring of known sex.

TABLE IV.—*Sex of progeny females*

Dam	Sex of offspring			Deviation—probable error
	Males	Females	Unknown	
5.1	14	17	4	0.8
12.2	30	16	5	3.06
21.2	17	21	12	.96
26.1	21	10	2	2.93
26.2	22	21	14	.22
26.4	11	15	4	1.16
54.1	14	14		
56.3	16	22	1	1.44
65.5	17	16	4	.26
65.6	17	13	8	1.08
68.5	12	18	3	1.62
293A2	13	12	1	.30
297.1	15	13	12	.56
369.2	14	16	5	.54
370.2	14	16	1	.54

If the observed irregularities in the sex ratio were due to the presence of sex-linked lethal factors in some of the breeding stock, the records of individual sires and dams should show this fact. Tables III and IV do show a number of irregularities, but perhaps no more than might be expected upon the basis of chance deviation. The breeding records of ♂316.1, ♀12.2 and ♀26.1 deviate just far enough from expectation that it is possible but not necessary to regard them as significant. All three animals are now dead and it is impossible to apply breeding tests to settle the question.

SEX IN RELATION TO DILUTION OF SEMINAL FLUID

The degree of dilution of the recovered liquid has an effect upon completeness of separation of the liquid and the solids contained in it, as already mentioned, but that it does not seem to have had an effect upon the sex ratio of the offspring produced is shown by Table V. The figures given for degree of dilution are in terms of the volume of Ringer's solution, which was added to the liquid recovered. Three volumes was the amount usually added, but for various reasons, usually to secure enough volume to be handled easily, other dilutions were sometimes made.

RELATION OF SPEED AND DURATION OF CENTRIFUGING TO SEX OF OFFSPRING

That the speed at which the centrifuging was done did not affect the sex of the offspring produced is shown by Table VI. The true speed of the electric centrifuge was undoubtedly somewhat greater than the 1,600 r. p. m. and the 2,000 r. p. m. measured, because the cover was on during the actual centrifuging, and therefore the air resistance was less than when the speed was being measured with a revolution counter.

TABLE V.—*Degree of dilution and sex of offspring*

Degree of dilution	Sex of offspring			Deviation—probable error
	Male	Female	Unknown	
None	32	28	1	0.77
1 to 2	21	27	5	1.28
3	79	92	8	1.47
4 to 5	16	12		1.12
8 to 12	13	4	1	3.23
Uncertain	22	11	4	2.84

TABLE VI.—*Speed of centrifuging and sex of offspring*

Speed	Sex of offspring			Deviation—probable error
	Male	Female	Unknown	
1,600 r. p. m. (hand centrifuge)	61	72	3	1.41
1,600 r. p. m. (electric centrifuge)	97	85	3	1.32
2,000 r. p. m. (electric centrifuge)	10	5		1.91
Odd and unknown speeds	4	6	1	.94

That the length of time the liquid was centrifuged is not a controlling factor is shown by Table VII, which gives the sexes of the rabbits produced from spermatozoa which had been centrifuged for varying lengths of time.

TABLE VII.—*Time of centrifuging and sex of offspring*

Time centrifuges	Sex of offspring			Deviation—probable error
	Male	Female	Unknown	
0 to 2 minutes	22	21		0.23
2 to 4 minutes	31	41	1	1.75
4 to 6 minutes	30	33	10	.56
6 to 10 minutes	75	62	6	1.65
10 to 15 minutes	7	5	6	.85
Unknown		4		2.97

MISCELLANEOUS FACTORS

The number of copulations as related to the sex of the offspring showed no effect, but the females were in excess in all classes except the four-copulation and five-copulation classes, in each of which the total number involved is less than 15. A large effect could scarcely be expected (*16*) because the number of copulations was in no case greater than five, and where there was more than one the liquid from the preceding copulations was mixed all together with that of the last, and therefore it is impossible to know whether a given one of the young was produced by a spermatozoon from the first or a later copulation.

It is hardly to be expected that the amounts of liquid recovered by the methods used in this experiment would be comparable enough to the amounts actually ejaculated to show a difference in the sex ratios produced by different amounts, even if such a difference existed. The sexes were either exactly even or there was a slight excess of females, which approached significance in only one case and did not seem to vary uniformly as related to the amount of liquid.

The microscopically distinguishable differences in the activity of the spermatozoa did not show any corresponding differences in the sex ratios of the offspring produced. This is not surprising, since no liquid was used for insemination unless there was at least a moderate number of fairly active spermatozoa to be seen in it.

RELATION OF TYPE OF BREEDING TO SUCCESS IN INDUCING PREGNANCY

The records kept before the centrifuging was begun were not detailed enough to permit taking any figures as to services which were natural in every way. Table VIII gives the number and percentage of services and inseminations which resulted in pregnancy and also the number which did not result in pregnancy. The percentage of success is figured only for those methods which were tried at least 30 times.

Several factors combine to make the data in Table VIII only approximate. A number of females would accept service while pregnant and a number were barren either through an infection in the Fallopian tubes or for some other reason. Such females were kept when there was room for them and were used for the recovery of seminal fluid, because the main object of the experiment was the production of the largest possible number of young by insemination with centrifuged or with cooled liquid, and so it was desired to use the fertile females for insemination. Therefore the true percentage of success for the natural-service type of breeding where the liquid was withdrawn is considerably higher than the figure given. The very low percentage of success where the fluid was cooled for some time before insemination brings down the percentage of success in the total inseminated, and in the inseminated but not centrifuged much lower than it should be in comparison to the

TABLE VIII.—*Relation of type of breeding to success in inducing pregnancy*

Type of breeding				Failed	Succeeded	Per cent of success
Natural service	Fluid withdrawn without washing			300	69	18.7
	Fluid washed out			10	7	
Inseminated	Normal	Undiluted		31	4	11.4
		Diluted		6	0	
		Delayed		1	1	
	Centrifuged	Undiluted	Whole sample	4	0	
			Inside sample	30	4	11.8
			Outside sample	23	9	28.1
		Diluted	Whole sample	1	0	
			Inside sample	172	35	16.9
			Outside sample	157	36	18.7
	Held some time at abnormal temperatures	Cold	Undiluted	79	3	3.7
			Diluted	55	5	8.3
		Warm	Undiluted	23	1	
			Diluted	6	0	
Total undiluted				190	21	10.0
Total diluted				397	76	16.1
Total inseminated				588	98	14.3
Total natural services with semen removed				310	76	19.7
Total centrifuged				387	84	17.8
Total inseminated but not centrifuged				201	14	6.5

natural service with semen removed or to the total centrifuged—that is, the apparent conclusion that centrifuging increases the chances of pregnancy over insemination without centrifuging is probably due only to the harmful treatment given the uncentrifuged fluid. Just why dilution of the liquid increased the chances of pregnancy is not known; the Ringer's solution may have stimulated the spermatozoa to greater activity or in some other way may have made fertilization more easy. Probably the absolute percentage of success should be higher in nearly every case, because some barren females were used which were not known to be barren and some females died or were sold while still pregnant.

The general conclusion can be drawn that spermatozoa retain their activity and fertilizing ability under more adverse conditions than is popularly supposed. Being kept in an ice box for eight days did not completely destroy their motility. Neither did the unusual handling involved in centrifuging and artificial insemination destroy their motility. Whether motility and fertilizing ability are the same thing is another question; but even though they are not, many young are produced from centrifuged spermatozoa and many were produced from spermatozoa exposed to low temperature for periods up to 24 hours. The only factor found to injure seriously the activity of the spermatozoa was the presence of urine in the liquid, and no experiments were made to determine whether this toxicity was due to acidity or to a toxic action of some other substance in the urine.

MONTHLY DISTRIBUTION OF LITTERS BORN IN LAST 10 YEARS

The breeding season of the rabbit under natural conditions is usually given as in the late winter or early spring. Table IX gives the number of litters by months which were born in the last 10 years in this flock.

These figures can be regarded only as giving an approximation to the true breeding season because so many other factors varied. Thus the breeding flock was much larger at some times than at others. Also the breeding work was pushed more vigorously some months than others, and during at least 3 years no breeding was done during July and August. The figures are presented as tending to support the idea that the breeding season is in the spring and late winter but denying that breeding is confined to that season.

TABLE IX.—*Monthly distribution of litters born in last 10 years*

Month	Number of litters	Month	Number of litters
January	28	July	29
February	44	August	21
March	75	September	14
April	66	October	15
May	89	November	21
June	49	December	40

OVULATION

As quoted by Marshall (*29*), Heape (*17*) maintains that ovulation in the rabbit is dependent upon the stimulus of coition and can not be induced by artificial stimulation, but Weil (*41*) maintains that ovulation may take place without coition just after the delivery of a litter, and Iwanoff (*19, 20*) induced pregnancy by artificial insemination of rabbits just after parturition. That neither of those views goes far enough is shown by the four litters produced in these experiments by artificial insemination of females which had neither delivered a litter nor copulated within less than 13 days previous and were not even placed with a male for 10 days following. A fifth litter was produced by a female which had been inseminated the day after she had produced a litter. She was evidently in heat but was not allowed to copulate. This is a parallel case to those of Iwanoff. It is certain that ovulation can take place without the stimulus of coition, but it is not yet certain that ovulation proceeds without any psychic stimulus from the courtship of the male as it does in the case of the cow. It may be that the stimulus of courtship is necessary, as it is in pigeons, even though it may be two females which court each other. In the case of these four litters the females were placed with the test males and showed signs of heat, but were removed before they could copulate; hence the physic stimulus of courtship was present; but that it was necessary seems improbable, since the females often show signs of heat immediately upon being placed with other females, mounting them and sometimes even having an orgasm when mounted, although no male is near and the female in heat has been alone in her hutch for 24 hours.

MICROSCOPIC WORK

The measurements of rabbit spermatozoa from seven different slides are shown in Figures 1 to 7, together with the theoretical normal curves for populations of those sizes with those means and standard deviations. Slides Nos. 370, 371, and 372 were all made from the same sample of recovered semen. Slide 277 was made from semen of the same buck but recovered at a different time.

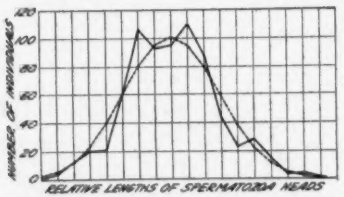

FIG. 1.—Actual (solid line) and theoretical (broken line) frequency distribution of the head lengths of untreated rabbit spermatozoa. The actual distribution is clearly bimodal and the probability that it is only a chance deviation from the theoretical distribution is less than 0.001. (Slide No. 370)

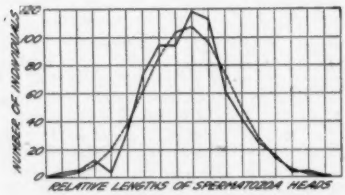

FIG. 2.—Actual (solid line) and theoretical (broken line) frequency distribution of the head lengths of rabbit spermatozoa taken from the inside of the centrifuge tube after being centrifuged 5 minutes at 1,600 r. p. m. The liquid had first been diluted with three times its volume of Ringer's solution. Made from the same sample of semen as that in Figures 1 and 3. The probability that the actual distribution is only a chance deviation from the theoretical normal curve is 0.207. (Slide No. 371)

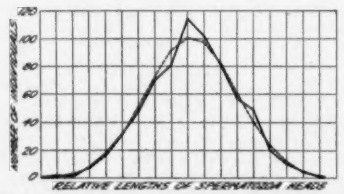

FIG. 3.—Actual (solid line) and theoretical (broken line) frequency distributions of the head lengths of rabbit spermatozoa taken from the outside of the centrifuge tube after being centrifuged five minutes at 1,600 r. p. m. The liquid had first been diluted with three times its volume of Ringer's solution. Made from the same sample of semen as that used in Figures 1 and 2. The probability that the actual distribution is only a chance deviation from the theoretical normal curve is 0.84. (Slide No. 372)

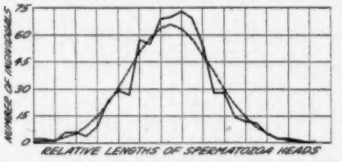

FIG. 4.—Actual (solid line) and theoretical (broken line) frequency distributions of the head lengths of untreated rabbit spermatozoa. The probability that the actual distribution is only a chance deviation from the theoretical is 0.12. (Slide No. 271)

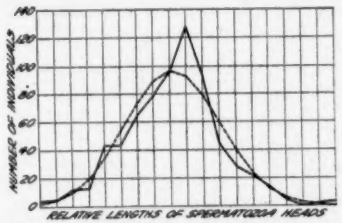

FIG. 5.—Actual (solid line) and theoretical (broken line) frequency distributions of the head lengths of untreated rabbit spermatozoa. The probability that the actual distribution is only a chance deviation from the theoretical is less than 0.001. (Slide No. 277)

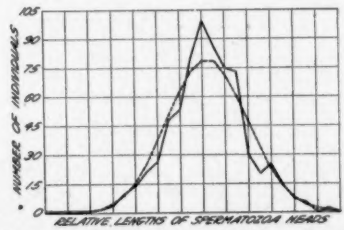

FIG. 6.—Actual (solid line) and theoretical (broken line) frequency distributions of the head lengths of untreated rabbit spermatozoa. The probability that the actual distribution is only a chance deviation from the theoretical is 0.029. (Slide No. 249)

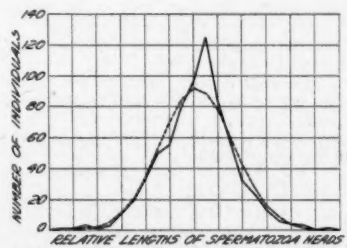

FIG. 7.—Actual (solid line) and theoretical (broken line) frequency distributions of the head lengths of centrifuged rabbit spermatozoa. From the outside portion of the centrifuge tube after being centrifuged eight minutes at 1,600 r. p. m. The probability that the actual distribution is only a chance deviation from the theoretical is 0.09. (Slide No. 263)

The measurements of swine spermatozoa from seven different slides are shown in Figures 8 to 14, together with the theoretical normal curves [6] for populations of those sizes with those means and standard deviations. Slides Nos. 519, 531, and 536 were made of semen out of the testicle of one boar, slides Nos. 522 and 526 were made from the fluid recovered from a natural service by the younger boar, and slide 537A was made of semen from the testicle of still a third boar. Two separate sets of measurements were made on slide 531 (figs. 10 and 11).

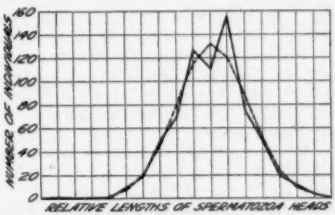

FIG. 8.—Actual (solid line) and theoretical (broken line) frequency distributions of the head lengths of untreated swine spermatozoa taken from the testicle of an old boar castrated three or four hours before this slide was prepared. The probability that the actual distribution is only a chance deviation from the theoretical is 0.017. (Slide No. 519)

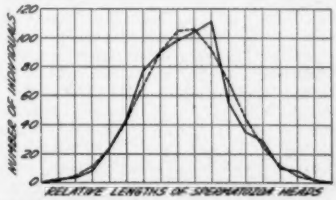

FIG. 9.—Actual (solid line) and theoretical (broken line) frequency distributions of the head lengths of centrifuged swine spermatozoa. From the extreme outside end of the centrifuge tube after five minutes of centrifuging at 1,600 r. p. m. From the same sample of semen used in preparing Figures 8, 10, and 11. The probability that the actual distribution is only a chance deviation from the theoretical is 0.31. (Slide No. 536)

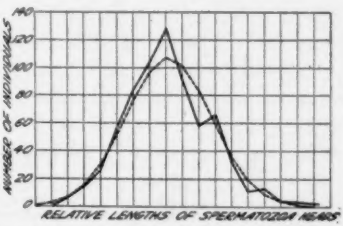

FIG. 10.—Actual (solid line) and theoretical (broken line) frequency distributions of the head lengths of centrifuged swine spermatozoa. From the extreme inside end of the centrifuge tube after being centrifuged five minutes at 1,600 r. p. m. From the same sample of semen used in preparing Figures 8 and 9 and from the same slide from which Figure 11 was prepared. The probability that the actual distribution is only a chance deviation from the theoretical distribution is 0.041. (Slide No. 531)

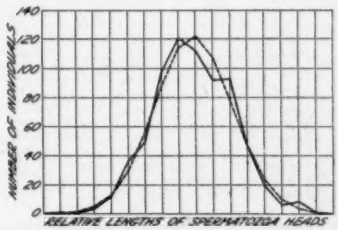

FIG. 11.—Actual (solid line) and theoretical (broken line) frequency distributions of the head lengths of centrifuged swine spermatozoa. From the extreme inside end of the centrifuge tube after five minutes of centrifuging at 1,600 r. p. m. From the same sample of semen used in preparing Figures 8 and 9 and from the same slide from which Figure 10 was prepared. Figures 10 and 11 were made from measurements made on spermatozoa from different parts of the same slide and thus serve as a control upon the accuracy of the measurements. The probability that the actual distribution is only a chance deviation from the theoretical distribution is 0.30. (Slide No. 531)

[6] The theoretical normal curves were constructed according to the method explained in West's "Introduction to Mathematical Statistics" (*42*). The curve is completely determined by three things—namely, its mean, standard deviation, and the number of individuals involved. These three figures for each theoretical curve are taken from its corresponding actual curve. The probability that the actual curve is only a chance deviation from the theoretical normal one is obtained by the X^2 method of testing the goodness of fit of one set of observed data to a corresponding set of expected data (*15*).

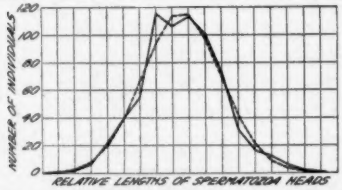

FIG. 12.—Actual (solid line) and theoretical (broken line) frequency distributions of the head lengths of untreated swine spermatozoa. Recovered from the vagina of a sow after natural service. The probability that the actual distribution is only a chance deviation from the theoretical is 0.26. This is from the same sample of semen as was used in the preparation of Figure 13. (Slide No. 522)

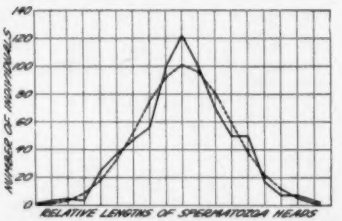

FIG. 13.—Actual (solid line) and theoretical (broken line) frequency distributions of centrifuged swine spermatozoa. From near the inside end of the centrifuge tube after five minutes of centrifuging at 1,600 r. p. m. From the same original sample of semen from which Figure 12 was made. The probability that the actual distribution was only a chance deviation from the theoretical distribution is 0.008. (Slide No. 526)

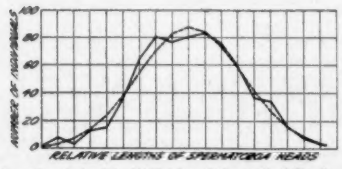

FIG. 14.—Actual (solid line) and theoretical (broken line) frequency distributions of untreated swine spermatozoa. The spermatozoa were obtained from the testicle of an aged Poland-China boar immediately after castration, and the milky fluid which was recovered from the epididymis was diluted with about six volumes of Ringer's solution and slide 537A was made directly from this. The probability that the actual distribution is only a chance deviation from the theroetical distribution is 0.55. (Slide No. 537A)

The microscopic work on the rabbit spermatozoa was directed toward the answering of two questions: First, whether there really are two different sizes of spermatozoa produced in approximately equal numbers, and second, whether the centrifuging tends to separate those two sizes. The answer to the first and more fundamental question is not certain. One of the four curves of normal spermatozoa is distinctly bimodal, one is distinctly unimodal, and the other two are broader at the top and narrower in the middle than the theoretical curve but agree with it well at the base. That is the result which would be given if the curve were a compound one composed of two equal normal curves whose modes were not very far apart. Since the size difference between the X-containing and Y-containing spermatozoa is expected to be very small (*1*), these flat-topped curves agree very well with the idea that there really are two sizes of rabbit spermatozoa, but that the means of those two sizes are too close together for the modes to show separately in the curves. All three of the curves of centrifuged spermatozoa are unimodal, and the two for which there is a control (figs. 2 and 3) seem to indicate in both cases that it is the larger spermatozoa which were separated out both to the inside and to the outside of the tube.

Those spermatozoa which were plainly distorted or broken were not measured, but the distortion of many of them may have been so slight that it could not be detected by inspection. Since the answer to the two principal inquiries is concerned only with the average distribution of the two classes of spermatozoa, the extreme variants have very little importance in this problem. Therefore, in figuring the goodness of fit of the actual results to the theoretical, the only classes considered were those in which the theoretical number exceeded six. This arbitrary limit includes the great majority of the individuals and gives a fair indication of the goodness of fit of the actual distribution to the expected distribution without giving undue importance to any chance variation where the expectation is small. It will be noticed that the probability is fairly high for all of the centrifuged slides as compared to the normal ones, there being only one normal one with a value as high as either of the three centrifuged slides. This indicates that the centrifuged samples are more homogeneous with respect to head length of spermatozoa than untreated samples are.

The general conclusions to be drawn from the microscopic work on the rabbit spermatozoa are that there are indications of a dimorphism in the head lengths but no evidence complete enough to establish it beyond question. The two sizes differ so

slightly that it is uncertain whether centrifuging effects a separation at all; certainly it is not a complete one, and the conditions under which each insemination and centrifuging are performed necessarily vary so much that the necessary adjustments of technique appear to be insurmountable difficulties to the successful control of sex in rabbits by this method even though the theoretical basis of the method may be perfectly sound.

The microscopic work on swine spermatozoa was directed toward answering the same two questions as was the work with the rabbits, but was unaccompanied by any breeding work. The first question, as to the existence of a dimorphism in the spermatozoa head lengths, has already been answered very positively by Wodsedalek (*43*), and since the difference reported between the mean sizes of the two classes was so large it seemed that this was excellent material for seeking an answer to the second question. However, the dimorphism revealed by the examination of untreated spermatozoa in this work was not nearly so distinct as that reported by Wodsedalek. Where the expectation is that the mean size of the larger kind will exceed that of the smaller kind by about 20 per cent, the actual results are that the mode of the larger kind is from 7.1 to 8.6 per cent larger than the mode of the smaller kind. Just what is the reason for the difference in results is not clear. The material used is almost identical, the animals being of the same breed, from the same herd, and one at least being of the same age as the one used by Wodsedalek. The only difference, aside from differences in staining and possible errors in measurement, is that the spermatozoa used in this work were mature spermatozoa from the epididymis of the testicles, or were already ejaculated, whereas Wodsedalek measured spermatozoa in the tubules themselves, using as a criterion of their maturity the fact that they were in the lumen free from the surrounding tubule walls.

However, it seems certain that a dimorphism of spermatozoa does exist, although it may not be as great as was first reported. All three of the curves of untreated spermatozoa show two modes, rather close together and of about equal height; all three are wider at the apex and narrower at the middle than the theoretical, and therefore, although the average figure for their goodness of fit is fairly high, it seems certain from inspection that they really are compounded of two overlapping curves of about equal frequency.

The slides made from the centrifuged material agree in indicating that centrifuging tends to separate the smaller spermatozoa to the inside and the larger spermatozoa to the outside of the centrifuge tube, but that the separation is not complete. All three of the slides made from the inside material are skewed to the left and the one made from the outside is quite strongly skewed to the right. The unusual shape of the curve from slide No. 526 may be connected with the fact that it was made of material from the next to the extreme inside one-sixth of the centrifuge tube instead of the extreme inside, as slide No. 531 was. Slides 519, 531, and 536 are made from the same material, and slides Nos. 522 and 526 are both made from material from the younger boar.

The conclusions which can be drawn from the work on swine spermatozoa are that there is a definite dimorphism in size, but that the difference between the two kinds of fully mature spermatozoa is probably not as great as was reported by Wodsedalek. Centrifuging effects a partial separation, throwing more of the large spermatozoa to the outside and leaving more of the small ones in the inside portions of the liquid. As pointed out in discussing the theory of centrifuging, this is the effect to be expected when bodies of varying size are suspended in a liquid which is slightly less dense than the bodies themselves, and that the spermatozoa are of a slightly greater density than the surrounding liquid is shown by the fact that the majority of the spermatozoa are thrown to the outside in 5 minutes of centrifuging and almost all of them if the centrifuging is prolonged much beyond 10 minutes.

Whether the separation is complete enough or the technique can be made simple enough so that sex control by a method based on these principles ever can be made practical in swine is still an open question. There does not appear to be much economic advantage in being able to control sex in swine, but the scientific interest would be considerable. Even if it can be made practicable in swine, such a method would be restricted to animals in which the X-chromosome constituted a very large and the Y-chromosome a very small percentage of the total chromatin of the cell. That, of course, eliminates a large number of animals, including

man himself, from the list of eligibles. In short, this investigation has not strengthened the belief that there is any method of controlling sex among the higher animals which is practical, simple, and of wide application.

SUMMARY

Centrifugal separation of the male-producing and female-producing spermatozoa was attempted with liquid recovered by means of a catheter from the vagina and uterus of female rabbits which had just been served by normal males. Other females were then artificially inseminated with portions of the centrifuged liquid or with liquid treated in other ways and the sex of the young thus produced was recorded. The only deviation from equality which approached statistical significance was in the sexes of the young produced by females which had been served naturally and then had had as much liquid as possible recovered from the vagina and uterus by means of a catheter. There is no evident explanation of this result, and since the deviation is barely significant statistically it is concluded that this is probably a chance deviation.

Microscopic measurements of the head lengths of a large number of rabbit spermatozoa, both before and after centrifuging, show that a dimorphism probably exists but that the size difference between the two groups is very small. It is not certain whether centrifuging has had any effect upon the distribution of sizes.

Similar measurements of swine spermatozoa have shown the existence of a dimorphism in size, but not as great a difference as was reported by Wodsedalek. Centrifuging effected a partial separation, tending to throw more of the larger spermatozoa to the outside of the tube and leaving more of the smaller spermatozoa near the inside.

Sex control by centrifugal separation of male-producing and female-producing spermatozoa seems possible for species where the difference in the sizes of the X-chromosome and the Y-chromosome is a considerable percentage of the total amount of chromatin in the spermatozoa. Whether such a method can be made practicable even for the most favorable species depends upon whether the technique can be simplified and made dependable.

There is nothing in this work which can be regarded as an argument against the chromosome theory of sex, but, on the other hand, there is no new evidence in support of it. The failure to control sex in rabbits may be tentatively attributed to the impossibility of refining the technique to the point where advantage could be taken of the very small difference which exists between the X-chromosomes and the Y-chromosomes of the rabbit.

Spermatozoa retained their fertilizing ability as long as 24 hours in vitro at low temperatures and retained their motility as long as 60 hours in vitro at moderately low temperatures and up to 200 hours when kept in an excised testicle on ice.

Coition is not necessary to ovulation in the rabbit.

The chances of a female becoming pregnant when inseminated were increased by diluting the fluid used, but were decreased by holding it some time at a low temperature.

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CORRELATED INHERITANCE OF BOTANICAL CHARACTERS IN BARLEY, AND MANNER OF REACTION TO HELMINTHOSPORIUM SATIVUM [1]

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INTRODUCTION

The identification of linkage groups and their relation to chromosome numbers is a subject of general genetic interest. In only one organism, Drosophila, have all of the linkage groups been thoroughly worked out. Studies are being made of other organisms, however, with the idea of identifying the chromosomes by the behavior of the factors which they carry. Much has been learned of the factor relations in maize, largely from the efforts of Emerson and his coworkers. It is obviously a more difficult task to establish 10 linkage groups, which is the number of chromosome pairs in corn, than it is to establish 4 such groups, as in Drosophila. For linkage studies, an organism with a small number of chromosomes is desirable and from this standpoint, perhaps, barley offers as favorable material as can be found among the crop plants. Furthermore, barley has a number of easily distinguishable characters.

Through the introduction of new plant varieties from foreign countries and as a result of extensive hybridization studies, new characters are frequently being made available, and some of these have considerable economic importance. A 6-rowed variety of barley with smooth awns and resistance to *Helminthosporium sativum* has been developed and promises to take the place of the best 6-rowed varieties now generally grown in Minnesota. No such 2-rowed variety is known, however. Since there is a demand for 2-rowed barleys in certain sections of Minnesota, the production of a 2-rowed variety with smooth awns and other desirable characters is of some economic importance.

REVIEW OF LITERATURE

A somewhat comprehensive review of literature on barley inheritance was made by Hayes and Garber (*8*).[3] The literature review in the present paper indicates the important work on barley inheritance in its various phases, but the discussion of that work has been somewhat limited on account of lack of space.

CYTOLOGICAL STUDIES

The chromosome number for barley was reported first by Nakao (*13*) and later by Ubisch (*23*, v. *25*). Nakao used a variety of *Hordeum distichon* and found the somatic number of chromosomes to be 14. The same number of chromosomes was found by Ubisch in varieties of *H. distichon* and *H. vulgare*.

INHERITANCE OF DIFFERENTIAL CHARACTERS

Fertility of the lateral florets.—The results obtained from crosses of 2-rowed with 6-rowed varieties vary. Hayes and Garber (*8*) state that "the most frequent result is an intermediate condition in F_1 in which the lateral florets are awned, but produce little or no fruitfulness. In F_2 a 1:2:1 ratio of 6-rowed, intermediate, and 2-rowed forms is obtained. Six-rowed and 2-rowed forms breed true to these respective characters in later generations. Results of this nature can easily be explained on a single main factor difference."

Some crosses of 6-rowed with 2-rowed forms yield different results from the above, however, as was shown by Harlan and Hayes (*6*). The 6-rowed parent carried two factors for fertility of the laterals, one for 6-rowed and another for intermedium which is hypostatic to the 6-rowed factor. There were indications that sometimes modifying factors were present which affected the degree of fruitfulness in the laterals of the intermedium form. Results of some crosses

[1] Received for publication July 3, 1924; issued July, 1925. A paper submitted to the faculty of the University of Minnesota in partial fulfillment of the requirements for the degree of doctor of philosophy. Published with the approval of the Experiment Station Director as Paper No. 479 of the Journal Series.

[2] The writer wishes to express his appreciation for the helpful suggestions of Drs. H. K. Hayes, E. C. Stakman, and C. O. Rosendahl. Thanks are also due J. J. Christensen for cultures of Helminthosporium, and to E. B. Lambert for assistance in taking some of the data.

[3] Reference is made by number (italic) to "Literature cited," p. 933.

indicated a single-factor difference between intermedium and 6-rowed. Crosses of deficiens with 2-rowed also showed a one-factor difference.

Arlington awnless, classed by Wiggans (*26*) as an intermedium, has been used by Saunders and Moe (*18*) as the female parent in a series of crosses. Arlington awnless×6-rowed bearded produced in F_1 an intermediate type and in F_2 Arlington awnless, intermediate, and 6-rowed in a 1 : 2 : 1 ratio. Arlington awnless×2-rowed hooded produced in F_1 a type resembling the female parent for fertility of the florets in the lateral spikelets but with the florets of the medians hooded. In the second generation the plants were grouped into four classes: I. 2-rowed hooded; II. 6-rowed, or approximately 6-rowed, without awns on the florets of lateral spikelets but with hoods on the florets of the median spikelets; III. 2-rowed bearded with occasionally a few kernels present in the lateral rows; and IV. Arlington awnless type. Plants from Class I bred true in F_3 for the 2-rowed condition but some families segregated for hoods and awns. The plants from Class II differed in the results they gave in F_3. Plants from Class III bred true for 2-rowed bearded. Plants from Class IV were of two types, one bred true for the Arlington awnless type and the other gave Arlington awnless, 2-rowed bearded, and intermediates.

AWNS AND HOODS.—According to Hayes and Garber (*8*), Tschermak obtained results showing a single-factor difference between awnless and hooded forms, with the awnless character dominant. In crosses of hooded varieties with awned varieties the hooded condition tended to be dominant, the ratio in F_2 being 3 hooded to 1 awned. The same results were obtained by Thatcher.

In a series of studies, Ubisch (*23*, *24*) found an interesting relation between the hooded and awned characters. He considered a spike as long-awned when the awns were 6 cm. long or longer, and as short-awned when the awns were shorter than 6 cm.

In later studies Ubisch (*24*) crossed awnless varieties with short-awned and in some cases obtained an awnless F_1 type and in others a hooded F_1 type. The results of the later generations have not been published.

ARTICULATION OF RACHIS.—In some varieties of barley, particularly of the species *Hordeum spontaneum*, the rachis is articulate and the heads shatter quite easily. A one-factor difference between the articulate and the nonarticulate rachis was found by Schiemann (*21*) in a cross in which *H. spontaneum* was used as one of the parents. Articulate types were obtained by Ubisch (*23*, *v. 17*) from crosses in which both the parent varieties were nonarticulate. The ratio of plants with articulate rachis to plants with nonarticulate rachis was approximately 9:7 in the F_2 generation. In one cross the articulation was as pronounced as that of *H. spontaneum*, but in the other crosses was less pronounced.

DENSE AND LAX SPIKE.—The terms dense and lax, as used here, refer to the length of the rachis internode. In dense varieties the internode is short, and in the lax varieties long. The terms lax and dense are relative, since homozygous forms apparently exist (*6*) with small gradations all the way from very dense to very lax. Several factors for density of the rachis were shown to be present in one cross, while other crosses differed apparently by only a single differential factor. Ubisch (*23*, *v. 17*) considered the forms with internode length of 3.5 mm. or greater as lax and with internode of less than 3.5 mm. as dense. With this grouping he obtained one main factor difference for dense versus lax. He assumed other factors with lesser effect were present in some crosses.

ROUGH AWN AND SMOOTH AWN.—In some varieties of barley the awn has sawlike teeth from base to tip. Various types of teeth were described by Vavilov (*25*), who made a careful morphological study of the teeth on a number of varieties. He also found various degrees of smoothness but failed to find any varieties which lack the teeth on the entire length of the awn. Crosses of rough-awned with smooth-awned varieties were first studied by Harlan (*5*), who found a single factor difference between rough awn and smooth with rough dominant. Vavilov obtained smooth-awned types in the F_2 generation from crosses in which both the parent varieties were rough-awned. In crosses of rough-awned varieties with smooth the F_1 plants were always rough-awned. Crosses were made between rough-awned and smooth-awned varieties at the Minnesota Experiment Station (*10*). The awns of the F_1 plants were rough. In the F_2 generation the ratio of rough to smooth awned plants was approximately 3:1. Third-generation lines from F_2 plants classed as rough-awned varied in their breeding behavior. Some lines produced only rough-awned plants, some produced both rough and smooth, and others

contained all the types of F_2. The results are explained on the basis of one main factor difference between rough awn and smooth awn.

TEETH ON THE GLUMES.—Three kinds of teeth on the glumes of barley are described by Ubisch (*23, v. 17*). Teeth of type (*a*) are visible to the naked eye, type (*b*) are seen by the aid of a hand lens, while type (*c*) can be seen only with a microscope. In crosses of type (*a*) with a nontoothed variety an intermediate toothed condition was obtained in F_1, and in the F_2 there were 3 toothed and intermediate forms to 1 nontoothed.

HULLED AND NAKED SEED.—The naked condition of the seed seems to be a simple recessive to the covered. Hayes and Garber (*8*) cite the results of Gaines and also Thatcher in which the naked condition behaved as a simple recessive to the covered. Ubisch (*23, v. 20*) obtained results which indicated that the hulled condition was not completely dominant, although the results could be explained on a single factor basis.

DWARF FORMS.—Harlan and Pope (*7*) describe a dwarf barley which measured only about 50 cm. from the crown of the plant to the tip of the awns. The dwarf form behaved as a simple recessive to the normal barley. On the other hand, a dwarf was obtained by Miyazawa (*12*) which tended to be dominant to the normal. The homozygous dwarfs were sometimes so small as to escape notice and were sterile. Occasionally a dwarf plant died in the seedling stage. A single factor difference explains the results obtained.

CHLOROPHYLL DEFICIENCES.—Seedling types deficient in chlorophyll have been found in several of the cereals, so it is not surprising to find that they also occur in barley. They have been observed in two Minnesota varieties, but their mode of inheritance has not been studied. Hallquist (*3*) obtained a chlorophyll-defective barley strain which is dependent on temperature for its expression. At 0° to 10° C. no chlorophyll develops, at 12° to 15° some green color develops but the seedlings are very yellow, while at 20° the seedlings are normal green in color almost from the time of their emergence. The defect behaves as a simple recessive to the normal green. Back crosses of the heterozygotes to the normal green gave results which differed depending on whether the normal was used as the female or as the male parent. The results are explained by assuming a single factor difference between the normal and the defective and a partial elimination of the male gametes which carry the recessive factor.

Six different chlorophyll defects are described by Nilsson-Ehle (*14, 16*), three whites, two yellows, and a chlorina. The three whites have been shown by breeding tests to be different genotypically. The yellows have not been tested thoroughly but differ phenotypically. The chlorina corresponds to the virescent types in corn in that under the proper cultural conditions it lives and produces seed. Each of the abnormal types behaves as a simple recessive to the normal green.

A mutation was discovered by Kiessling (*11*) which differed strikingly from the parent variety in many morphological and physiological characters, including a light green color of the foliage. The deviations from the normal, other than that of chlorophyll, are considered by Kiessling as incidental to the chlorophyll deficiency. The abnormal type, like those studied by Nilsson-Ehle, is a simple recessive to the normal.

DISEASE RESISTANCE.—Nilsson-Ehle (*15*) made a study of the inheritance in barley of the manner of reaction to the nematode disease caused by *Heterodera schachtii*. The cross Chavalier × Gold, immune and susceptible varieties, respectively, gave F_1 plants which were immune and the segregation in F_2, as shown by the breeding behavior in F_3, approximated a 3:1 ratio. These results show susceptibility to be a simple recessive to immunity.

Studies have been made by Hayes et al (*10*) on the inheritance of the manner of reaction in barley to *Helminthosporium sativum*. A careful study was made in a cross of a resistant variety with a highly susceptible variety. One hundred and twenty-four F_3 lines were grown under epidemic conditions in comparison with the parent varieties. The number of homozygous resistant and susceptible lines was determined from a test of F_3 and F_4 families. It was pointed out that the results could be explained by assuming two pairs of factors to be involved. The presence of both factors leads to the production of highly resistant forms while the absence of both gives highly susceptible forms.

COLOR IN GLUMES.—Black color in the glumes behaves as a dominant to white, and in all results so far obtained a single factor difference exists between black and white. Purple color in the glumes is also dominant to white, and

in crosses of purple with white the F_2 generation shows a ratio of 3 purple to 1 white.

LINKAGE RELATIONS.—Although considerable work has been done on inheritance in barley, comparatively few cases of linkage have been shown definitely. Ubisch (*23 v. 17, 20*) showed a correlation between the internode length of the rachis and the length of the awn. In crosses of varieties with long awns and lax spike with varieties having short awns and dense spike the F_2 results indicated a gametic ratio of 4 or 5 to 1. In later studies (*23, v. 25*) he obtained a correlation between the length of awn and naked seed. The gametic ration was 1:6. A gametic ratio of 1:5 was obtained in the cross of varieties having lax spike and naked seed with varieties having dense spike and covered seed. From these results, Ubisch reached the following conclusions: Factors for the length of awn and the length of internode are linked with 16.7 per cent crossovers; factors for the length of awn and covered versus naked seed are linked with 14.3 per cent crossovers; and factors for internode length and for covered versus naked seed are linked with 16.7 per cent crossovers. These results seem inconsistent with the linear theory for the arrangement of genes in the chromosome. Several factors have been found, however, producing varying densities of the spike. Because of this fact and the absence of a careful statistical analysis one is hardly justified in drawing definite conclusions from these results. Ubisch (*23, v. 17*) also found a linkage between the factor for 6-rowed with one of the factors for teeth on the glumes. The crossover percentage was 16.7.

A correlation was found by Vavilov (*25*) between the factor for rough awn and the factor for naked seed. The intensity of the linkage was not worked out.

Nilsson-Ehle (*16*) showed that one of the factor pairs for green versus white seedlings (factor pair Cc) is correlated with the factor pair for green versus chlorina (factor pair Ff). In the cross CcFF with CCff he studied the progeny of the combination cF×Cf. In F_2 he expected a ratio of 9 green : 3 chlorina : 4 white, and obtained 8.44 : 3.94 : 3.62, respectively. Instead of the normal 1 : 2 : 2 : 4 ratio in F_3 from green F_2 plants, he obtained no families which bred true for green, 9 families which segregated into green and chlorina, 12 which segregated for green and white seedlings, and 126 families which produced all types. He suggested linkage with about 10 per cent crossing over as the best explanation.

In the foregoing review a number of character pairs are considered. It seems, however, that little has been done to establish the relation of these character pairs to each other. One of the best studies from the standpoint of the relation of factors for several character pairs is that carried on at the Minnesota station in cooperation with the Office of Cereal Investigations, Bureau of Plant Industry, United States Department of Agriculture, and cited by Hayes and Garber (*8*). Four character pairs were studied in one cross: Hooded versus awned, 2-rowed versus 6-rowed, black versus white seed, and hulled versus naked seed. These four character pairs were shown to be independently inherited or, in other words, to represent four linkage groups.

According to the work of Vavilov (*25*), one of the factors for dentition of the awn is correlated with the factors for hulled versus naked seed. The results obtained by Ubisch (*22*) indicated that this group also contains a factor for length of awn and one for length of rachis internode.

In the present study the various character pairs are considered in their relation to each other with an idea of establishing a linkage group for each chromosome pair.

MATERIAL USED

All varieties used in these studies are known to be pure lines:

Svanhals is a variety of *Hordeum distichon*. It has white glumes, rough awns, headed about July 6, and is highly resistant to the spot blotch disease caused by *Helminthosporium sativum*.

Lion is a variety of *Hordeum vulgare*. It has black glumes, smooth awns, headed about June 21, and is highly susceptible to the spot blotch disease.

Manchuria, I–16–66, is a selection from Manchuria, a variety of *Hordeum vulgare*. It has white glumes.

Hordeum deficiens steudelii, as its name indicates is a *deficiens* variety. It has black glumes and the lemma and palet of the florets in the lateral spikelets are developed slightly or not at all.

Hordeum intermedium cornutum is a variety of *intermedium* (*4*) with practically complete fertility in the florets of the lateral spikelets. The florets of the median spikelets are hooded. It is hulless and has white seeds.

The results given here are from the crosses Svanhals×Lion and *Hordeum deficiens steudelii*×Manchuria. The crosses were made at University Farm, St. Paul, Minn., in 1921 and the F_1

generations were grown in the United States Department of Agriculture greenhouse at Arlington Experiment Farm, Rosslyn, Va., in the winter of 1921–22 and also in the plant breeding nursery at University Farm in 1922. Second generations were grown at University Farm in 1922 and 1923. Progeny from the 1922 F_2 generation plants were tested for breeding behavior in 1923.

EXPERIMENTAL RESULTS

In the presentation of the experimental results the data will be considered only for those characters for which the results seemingly warrant rather definite conclusions.

CYTOLOGICAL STUDY

So far as the writer is aware, no other work has been done in America on the chromosome numbers in barley species. The reports of Nakao and Ubisch are incomplete, not all cultivated species being included.

The photomicrographs included here are from transverse sections of the root tips of barley. The killing fluid used is a modification of Bouin's (used first by Allen (*1*) and recently used successfully by Sax [4]) on wheat material at the Maine Agricultural Experiment Station. The sections were cut 5 μ thick and stained with Haidenhain's iron alum haematoxylin.

Since crosses of varieties of the four cultivated species show no sterility and the chromosome number of some species was shown to be 14 in the somatic cells, one naturally would expect to find the same number in all cultivated species. A variety of each of the species *Hordeum vulgare*, *H. intermedium*, *H. distichon*, and *H. deficiens* is included in the cytological study. The chromosome number is the same for all varieties studied—that is, 14 in the somatic cells. This fact leads one to conclude that seven linkage groups will be found. On the basis of chromosome numbers, all varieties of these four species belong in one group (pls. 1 and 2).

As the writer is interested mainly in chromosome number from the standpoint of linkage groups, and since the photomicrographs show the number clearly, no drawings were made. One peculiarity of structure seems worth noting, however (pl. 1, A and B), and that is a more or less definite area near the end of a chromosome which does not take the stain. That the piece beyond the hyaline area is a part of the chromosome back of it is indicated by the fact that the small piece is always at the end and in line with the main part of the chromosome. When the chromosomes are long and thin (as in pl. 1, C), usually two chromosomes of the group have this disjointed appearance.

INHERITANCE OF DIFFERENTIAL CHARACTER PAIRS

FERTILITY OF LATERAL FLORETS.—In the cross *H. deficiens steudelii*× *Manchuria* the F_1 plants which were grown in the greenhouse could not be distinguished from the *deficiens* parent. Under field conditions, however, many F_1 plants show slight development of the glumes and palets in the laterals, and in some seasons the F_1 may be quite accurately distinguished from the homozygous deficiens. In the F_2 generation, grown in 1922, the plants could be grouped quite easily into the three types, *deficiens*, intermediate, and 6-rowed, and approximated closely a 1:2:1 ratio (Table I).

TABLE I.—*Classification of F_2 plants for the characters, deficiens and vulgare in the cross Hordeum deficiens steudelii*× *Manchuria (vulgare); 1922*

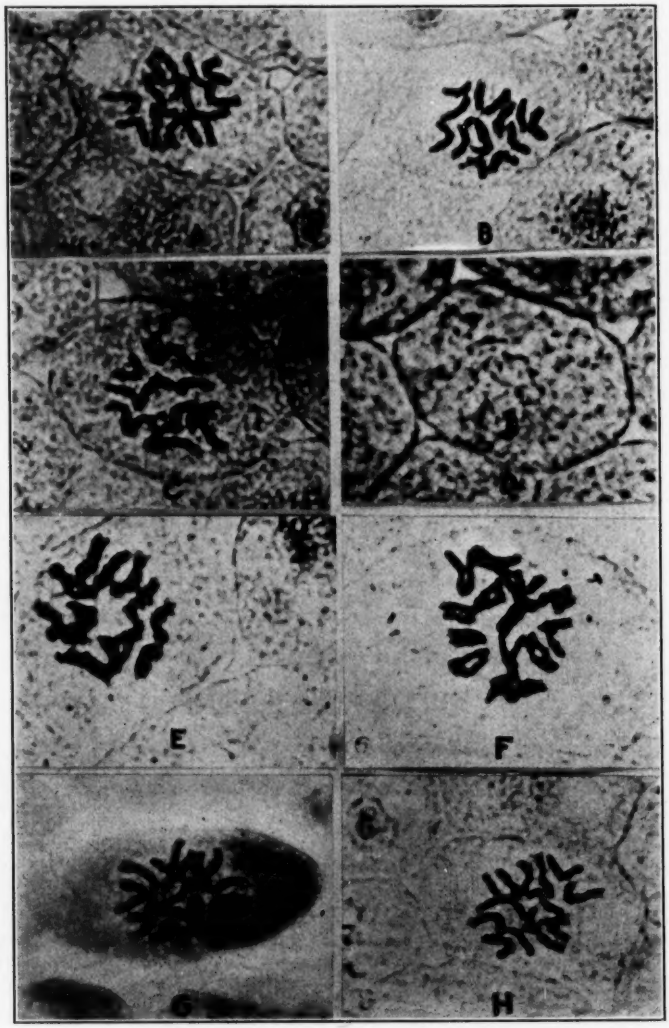
Class			Total
Vulgare	Intermediate	Deficiens	
84	191	80	355 observed.
89	178	89	356 calculated on 1 : 2 : 1 basis.

X^2=2.14. P=0.3476.

All plants classed as 6-rowed in F_2 bred true for this character in F_3 (Table II). All of the *deficiens* plants tested bred true, while all of the plants classed as intermediate segregated with one exception.

The heterozygotes and deficiens could not always be distinguished in the F_2 generation which was grown in 1923, but when these two groups are considered together the resuts show a good fit for a 3 : 1 ratio of deficiens and intermediates to 6-rowed (see Table III). These results show conclusively that the characters 6-rowed and de-

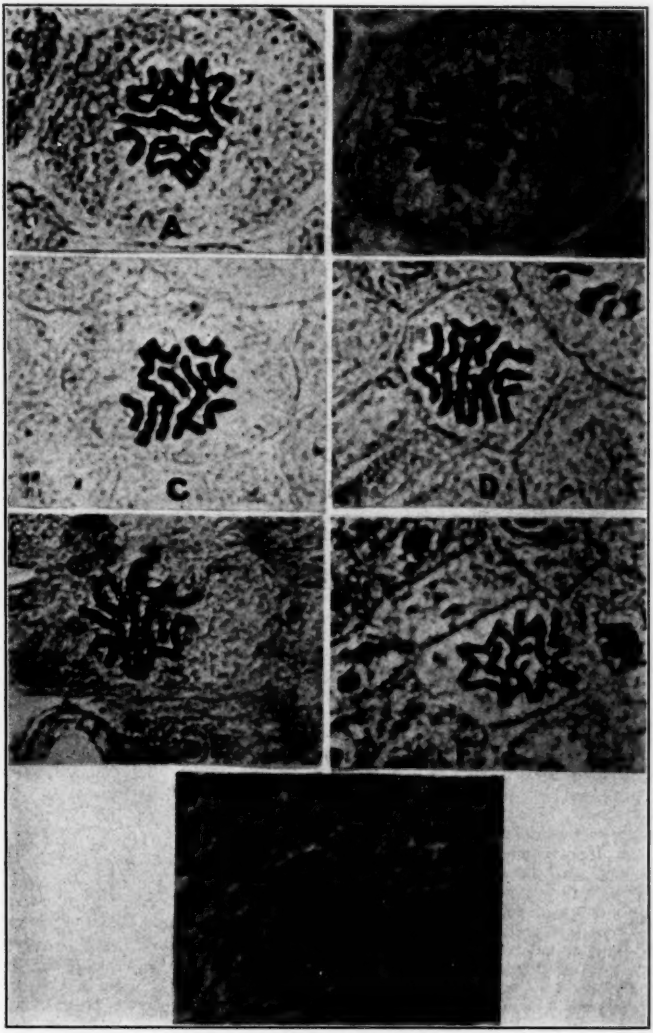
[4] Sax kindly furnished the writer with the details of his method of using Bouin's killing fluid.



A to F.—Cells from the root tips of Manchuria, a variety of *Hordeum vulgare*. In A, 14 chromosomes are clearly visible. In B, 12 chromosomes are clearly seen; the other two overlap at the ends and appear to be one big chromosome. C and D are different sections of the same cell, 13 of the chromosomes appearing in C and the other one in D. In E may be seen the beginning of the separation of the daughter chromosomes. In F is seen a later stage than that in E. × approximately 1,400

G and H.—These are from the root tips of *H. Intermedium cornutum*. Each contains 14 chromosomes. × approximately 1,400

(Material was killed in modified Bouin's killing fluid and stained with Haidenhain's iron alum haematoxylin. Sections were cut transversely in thickness of 5 microns. Unless otherwise stated, a K–1 filter was used in taking the photomicrographs)



A to D are from the root tips of Svanhals, a variety of *Hordeum distichon*. A and B are of the same cell. B was taken using a ground-glass filter with a 10-minute exposure, to bring out all details clearly

E to G are from the root tips of *Hordeum deficiens steudelii*. In E the 14 chromosomes are clearly visible. F shows only 13 chromosomes, but these are arranged in a peculiar pattern

(Material was killed in modified Bouin's killing fluid and stained with Haidenhain's iron alum haematoxylin. Sections were cut transversely, in thickness of 5 microns. Unless otherwise stated, a K–1 filter was used in taking the photomicrographs)

TABLE II.—*Behavior of F_3 lines from the three F_2 classes deficiens, intermediate and vulgare, in the cross Hordeum deficiens steudelii × Manchuria; 1923*

Type of plant	Number of plants tested	Classes in F_3			Number of families
		Deficiens	Intermediate	Vulgare	
Vulgare	20			×	20
Deficiens	32	×			32
Intermediate	50	×	×	×	49
		×			1

ficiens differ in a single genetic factor. The 6-rowed character may be considered to be due to the factor V, the 6-rowed condition being developed only when the plant is homozygous for this factor. The genotype for deficiens would then be vv.

TABLE III.—*Classification of F_2 plants for the characters deficiens and vulgare in the cross Hordeum deficiens steudelii × Manchuria; 1923*

Class			Total
Vulgare	Intermediate	Deficiens	
147	206	255	608 observed.
152	304	152	608 calculated on a 1 : 2 : 1 basis.
147	461		608 observed.
152	456		608 calculated on a 1 : 3 basis.

Deviation=5. P. E.=7.2.

Svanhals crossed with Lion gave F_1 plants which resembled the 2-rowed parent, but the glumes of the laterals were more pointed, and in some cases bore short awns. In the second generation the plants were grouped into four groups, 6-rowed, 2-rowed, intermedium, and intermediate. Only the 2-rowed and 6-rowed groups were tested thoroughly in F_3. All of the plants classed as 6-rowed bred true for that character. Fully two-thirds of the plants classed as 2-rowed in F_2 segregated in F_3; some produced 2-rowed, intermediates, and 6-rowed, some 2-rowed and intermedium, while others produced all types of F_2. The intermediums obtained in F_2 are of the same type as those obtained by Harlan and Hayes (*6*). Strains of this low fertility intermedium were obtained from the cross Svanhals × Lion, which bred true in F_3. Since all groups of F_2 plants were not tested thoroughly in F_3, a complete factor explanation will not be attempted. It should be pointed out, however, that the 6-rowed F_2 plants constitute approximately one-fourth of the entire number of plants (see Table IV), and that lines which segregated for 2-rowed versus 6-rowed gave a close approximation to a ratio of three 2-rowed and intermediates to one 6-rowed (see Table V). These facts, together with the appearance of a low fertility intermedium, indicate a two-factor explanation as used by Harlan and Hayes (*6*). These results prove that the low fertility intermedium factor may be carried by a 6-rowed variety and that the presence of this factor does not modify the expression of the factor for the 6-rowed condition. Two-rowed lacks the factors for intermedium and 6-rowed. Apparently the factor pairs which differentiate 6-rowed versus 2-rowed and intermedium versus 2-rowed are independently inherited.

TABLE IV.—*Distribution of plants for characters 2-rowed and 6-rowed in the F_2 generation in the cross Svanhals × Lion*

Year	Two-rowed and intermediates	Six-rowed	Total
1922	940	319	1,259 observed.
	944.25	314.75	1,259 calculated on a 3:1 basis.
	----------	4.25	Deviation.
	----------	10.30	Probable error.
1923	368	112	480 observed.
	360	120	480 calculated on a 3:1 basis.
	----------	8	Deviation.
	----------	6.4	Probable error.

TABLE V.—*Distribution of plants for the characters 2-rowed and 6-rowed in the F_3 lines which segregated, giving only these types and intermediates; cross Svanhals × Lion*

Two-rowed and intermediates	Six-rowed	Total
33	12	45.
27	13	40.
35	9	44.
34	13	47.
129	47	176 total observed.
132	44	176 calculated.
	3	Deviation.
	3.87	Probable error.

TABLE VI.—*Distribution of plants for the characters black glume and white glume in the F_2 generation of the crosses Hordeum deficiens steudelii × Manchuria and Svanhals × Lion*

Year grown	Black-glumed	White-glumed	Total	Cross
1922	263	92	355 observed	H. deficiens × Manchuria.
	266. 25	88. 75	355 calculated	
		3. 25	Deviation	
		5. 5	Probable error	
1923	470	138	608 observed	
	456	152	608 calculated	
		14	Deviation	
		7. 2	Probable error	
1922	871	283	1, 154 observed	Svanhals × Lion.
	865. 5	288. 5	1, 154 alculated	
		5. 5	Deviation	
		9. 9	Probable error	
1923	360	120	480 observed	
	360	120	480 calculated	
		0	Deviation	
		6. 4	Probable error	

TABLE VII.—*Behavior of F_3 lines from plants classified in F_2 as black glumed, in the crosses Hordeum deficiens steudelii × Manchuria and Savanhals × Lion*

Breeding true for black	Segregating in 3:1 ratio	Total	Cross
24. 0	41. 0	65. 0 observed	H. deficiens steudelii × Manchuria.
21. 7	43. 4	65. 1 calculated	
	2. 4	Deviation	
	2. 6	Probable error	
25. 0	58. 0	83. 0 observed	Svanhals × Lion.
27. 7	55. 4	83. 1 calculated	
	2. 6	Deviation	
	2. 9	Probable error	

BLACK VERSUS WHITE GLUMES.—The glumes of plants of the F_1 generation in the crosses *Hordeum deficiens steudelii* × Manchuria and Svanhals × Lion are as black as the colored parent. In the second generation the ratio of plants with black color to plants that are white is very close to 3 : 1 (see Table VI). All white F_2 plants bred true for white in F_3, as expected. Sixty-five F_3 lines from one cross and 83 from the other were grown from plants classed as black in F_2. The deviation from the expected 1 : 2 ratio is less than the probable error in each case (see Table VII). Apparently black and white differ by a single factor pair.

ROUGH AWN VERSUS SMOOTH.—Rough-awned varieties of barley are very disagreeable to handle, and for this reason the smooth-awn character is of considerable practical importance for those regions where awned varieties are preferred. The cross Svanhals × Lion gave F_1 plants with awns as rough as those of the Svanhals parent. The F_2 plants were easily classified into two groups, the first with awns entirely rough and the second with awns partially or almost entirely smooth. All plants which had awns with any degree of smoothness were studied carefully, the awns being examined under magnification. An index for smoothness was derived in a similar manner as in previous studies at the Minnesota station (*10*) which briefly is as follows: The total length of the awn in centimeters is divided by the portion of the awn from the tip where there are teeth of large size at frequent intervals. The higher the index of smoothness the smoother the awn, and vice versa. A rough awn has an index of 1 while the Lion parent used in this study has an average index slightly above 3. Some plants of Lion have a few scattered teeth, about half the distance from the tip to the base of the awn, but these teeth are usually of slightly reduced size.

An arbitrary division was made at an index of 2 and plants with a higher index were called smooth while plants with a lower index than 2 but with some degree of smoothness were called

"intermediate-smooth." With this grouping the distribution of plants in F_2 indicates a 2-factor difference for roughness of awn between Lion and Svanhals. The factor R, when present, gives rough awn. The other factor, S, is hypostatic to R and in the absence of R produces intermediate-smooth awn. The double recessive, rrss, is a smooth awn of the Lion type. With this explanation the ratio in F_2 would be 12 rough : 3 intermediate-smooth : 1 smooth. The observed distribution of plants in the F_2 generation grown in 1922 does not fit the calculated distribution very well, a deviation as large as that encountered being expected only about 2 times out of 100 trials (Table VIII).

The fit is better for the distribution of F_2 plants which were grown in 1923, but still the deviation is rather large. The reason for these deviations will be seen by examining Tables IX, X, and XI.

The behavior of the F_3 lines from plants classed in F_2 as rough is in close agreement with expectation, X^2 being 1.4 (P=0.7097). Of 37 F_3 lines from plants classed in F_2 as intermediate-smooth, 7 gave rough-awn plants in addition to intermediate-smooths, and smooths. However, the discarding of these families should not affect the expected 1:2 ratio of homozygous to heterozygous intermediate - smooths. Disregarding these 7 families, the other 30 families give a close approximation of a 1:2 ratio for homozygous intermediate-smooth to heterozygous (see Table X).

Thirty-four F_3 lines were grown from plants classed as smooth in F_2. Of these lines, 2 gave rough-awn plants, intermediate-smooth awn and smooth; while 8 gave intermediate-smooth and smooth. The other 24 lines vary in their indices from 2.85 to 3.61 (see Table XI). When a correction is made of the F_2 distribution of 1922, on the

TABLE VIII.—*Distribution of plants for the characters rough awn, intermediate-smooth awn, and smooth awn. F_2 generation of the cross Svanhals × Lion.*

Year grown	Character of awn			Total
	Rough	Intermediate-smooth	Smooth	
1922	908	251	100	1,259 observed.
	948	237	79	1,259+ calculated on 12:3:1 basis.
				X^2=8.1.
				P=0.0174.
1923	362	79	39	480 observed.
	360	90	30	480 calculated on 12:3:1 basis.
				X^2=4.0.
				P=0.1353.
Assumed genotype per 16.	1 RR SS ; 2 Rr SS	1 rr SS	1 rr ss	
	2 RR Ss	2 rr Ss		
	4 Rr Ss			
	1 RR ss			
	2 Rr ss			

TABLE IX.—*Summary of the behavior of F_3 lines from plants classified in F_2 as rough awn; cross Svanhals × Lion*

Behavior of lines	Number of families		Summary of lines segregating	P. E. or X^2
	Calculated	Observed		
Breeding true for rough	21.1	23		
Segregating rough : intermediate-smooth	10.6	9	290 : 71 observed 270 : 90 calculated	5.6
Segregating rough : smooth	10.6	8	262 : 81 observed 258 : 86 calculated	5.4
Segregating rough : intermediate-smooth : smooth	21.2	24	732 : 185 : 63 observed 732 : 183 : 61 calculated	X^2=1−
Total number families	63.6	64		

basis of the breeding behavior of F_3 lines, the observed frequency fits the calculated very well, P being 0.5278 (Table XII).

After two years' experience the writer is convinced that it is a simple matter to separate rough-awn plants from smooth and intermediate-smooth awn plants. The breeding test, however, is the only sure way to separate the intermediate-smooth and smooth-awn types.

Although a two-factor explanation is given to the results, considerable variability exists in the average indices of the F_3 lines breeding true for smooth. Row 75, for example, with an index of 3.61 ± 0.06 (see Table XI), differs markedly from row 63 which has an index of 2.85 ± 0.05. The difference between these two lines is 0.76 ± 0.08, which is certainly significant in the light of its probable error. These minor variations suggest the presence

TABLE X.—*Summary of the behavior of F_3 families from plants classed as intermediate-smooth in F_2; cross Svanhals × Lion*

Behavior of lines	Number of families		Summation of lines segregating	P. E.
	Calculated	Observed		
Breeding true for intermediate-smooth	10	12		
Segregating intermediate-smooth : smooth	20	18	558 : 176 observed 551 : 183 calculated	7.9

Seven lines gave rough, intermediate-smooth, and smooth.

TABLE XI.—*Behavior of F_3 families from plants classified in F_2 as smooth in comparison with the Lion parent; cross Svanhals × Lion*

F_2 Plant No.	1923 Row No.	Index of F_2 plant	Frequency for awn index of F_3 family				Total number plants	Average awn index	Standard deviation
			2.1	3.0	3.9	4.8			
Lion			6	25	12		43	3.13±0.06	0.57±0.04
Do			9	21	8		38	2.98± .07	.60± .05
Do			4	31	5	1	41	3.06± .05	.50± .04
Do			11	19	12	2	44	3.01± .08	.75± .05
Do			5	31	9		45	3.08± .05	.50± .04
Do			8	27	5		40	2.93± .05	.51± .04
Do			4	27	10		41	3.13± .05	.46± .03
Do			5	22	12		39	3.16± .06	.57± .04
Do			11	22	13	1	47	3.08± .07	.69± .05
Do			7	22	12		41	3.01± .06	.61± .05
Do			11	25	9		45	2.96± .06	.60± .04
Do			9	28	6		43	2.94± .05	.53± .04
Do			5	26	9		40	3.09± .06	.52± .04
Do			8	21	6	1	36	3.00± .07	.64± .05
115-164	63	3.3	11	26	4		41	2.85± .05	.52± .04
115-200	64	3.3	9	23	7		39	2.95± .05	.48± .04
139-172	68	3.3	8	25	11		44	3.06± .06	.59± .04
148-162	70	2.5	11	22	5		38	2.86± .06	.57± .04
148-163	71	3.3	6	25	6		37	3.00± .06	.51± .04
139-126	72	5.0	1	18	13	5	37	3.45± .06	.55± .04
115-195	75	3.3		13	15	6	34	3.61± .06	.49± .04
124-16	76	4.0	2	19	20	2	43	3.43± .06	.54± .04
130-4	77	3.3	2	31	7		40	3.11± .04	.51± .04
130-36	78	3.3	8	18	9	1	36	3.06± .07	.65± .05
130-146	79	4.0	3	18	19		40	3.36± .06	.59± .04
107-87	140	3.3	3	30	6		39	3.07± .05	.43± .03
107-117	141	3.3	4	20	12	1	37	3.24± .07	.62± .05
115-146	142	4.0	6	26	4	1	37	3.00± .06	.55± .04
115-156	143	3.3	11	25	5		41	2.87± .06	.55± .04
124-128	148	4.0	4	26	16		46	3.23± .05	.55± .04
130-129	149	3.3	2	18	15	2	37	3.41± .07	.62± .05
139-35	151	3.3	11	24	5		40	2.86± .06	.55± .04
148-25	153	2.8	8	26	8	1	43	3.04± .06	.61± .04
148-29	154	2.0	4	24	14	1	43	3.25± .06	.59± .04
148-244	155	3.3	9	25	6	1	41	3.09± .05	.52± .04
115-12	159	3.3	1	18	13	4	36	3.50± .07	.65± .05
130-187	162	3.3	5	21	14	1	41	3.24± .07	.63± .05
130-166	65	2.8	4	9	14		27	3.33± .08	.65± .05

TABLE XII.—*Distribution of F_2 plants for the characters rough awn, intermediate-smooth awn, and smooth awn, on the basis of breeding behavior in F_3; cross Svanhals×Lion, 1922*

F_2 class	Distribution on basis of F_3 behavior			Total
	Rough awn	Intermediate-smooth awn	Smooth awn	
	Per cent	*Per cent*	*Per cent*	*Per cent*
Rough awn	100			100
Intermediate-smooth awn	18.9	81.1		100
Smooth awn	5.9	23.5	70.6	100
Observed frequency	908	251	100	1,259
Corrected frequency	961	228	71	1,260
Calculated frequency on 12:3:1 basis	948	237	79	1,264
				X^2=1.33.
				P =0.5278.

of other factors affecting the distribution of teeth on the awn. On account of the difficulty encountered in an attempt to analyze characters of this nature, a more careful study than the one made is not considered feasible at the present time. When all of the linkage groups have been identified, it should be possible to locate easily these minor factors for dentition of the awn.

DATE OF HEADING.[5]—A study was made of the date of heading in 135 F_3 families of the cross Svanhals×Lion. The date of heading for Lion averaged about June 21 while that for Svanhals was around July 6, a difference of 15 days. (See Table XXVI, Appendix.) The F_3 lines proved to be of three kinds, as shown by the distribution for date of heading, two with a unimodal distribution and one with a bimodal. One type was like Svanhals, one like Lion, while the third produced both early and late plants with a preponderance of plants heading early. These three types appeared in approximately a 1:2:1 ratio. (Tables XIII and XXVI. The last named is in the Appendix.) The results show a one-factor difference for date of heading between Lion and Svanhals, early heading tending to be the dominant character.

REACTION TO HELMINTHOSPORIUM SATIVUM.—As already mentioned, Svanhals is highly resistant and Lion very susceptible to the spot blotch disease. For a study of the inheritance of the reactions to this disease, F_3 lines from the cross Svanhals×Lion were used. Seventy lines from plants classed as 6-rowed in F_2 and 65 lines from plants classed as 2-rowed were grown in F_3, making 135 lines in all. Among these lines the characters black and white, as well as rough awn and smooth awn, were represented. Twenty-five seeds of each line were planted in a 5-foot row and two systematically distributed rows were grown of each F_3 line. The parent varities were planted every tenth row. Inoculum was prepared by culturing the pathogene on a cooked wheat and barley mixture. It was sown in the rows at planting, and at heading time the plants were sprayed several times with a water suspension of spores.

TABLE XIII.—*Distribution of F_3 families for date of heading; cross Svanhals×Lion, 1923*

Early	Segregating	Late	Total
33	71	30	134 observed.
33.5	67	33.5	134 calculated on 1:2:1 basis.

X^2=less than 1. P=good fit.

Notes were taken on the infection by the same method as was used in previous studies at the Minnesota station (*10*). By this method a numerical figure is obtained which is an index of the reaction of the line, the higher the figure the more resistant the line, and vice versa. The notes were taken on the lines in the replicate rows without knowing what figure had been given them in the first series. The numerical figures for series one and series two were then correlated (Table XIV). The value of r was found to be $+0.382\pm0.050$.

[5] The time of awn emergency is taken as the most convenient criterion of date of heading.

TABLE XIV.—*Correlation of the Helminthosporium figure in series I and II of 135 F_3 families; cross Svanhals×Lion, 1923*

Figure for Series I	Figure for Series II							Total
	9	12	15	18	21	24	27	
9		1						1
12		1	7	4	1			13
15		2	11	11	4	3		31
18			6	11	6	5	1	29
21			5	20	15	5		45
24			1	4	3	5		14
27			1	1				2
Total		4	31	51	29	18	2	135

$r = +0.382 \pm 0.050$.

This figure, while not high, shows that definite differences exist between the F_3 lines and that these differences tend to appear alike in both series. The probable error calculated by the "deviation from the mean" method (*9*) was 4.7 per cent. With the probable error of the experiment as 4.7 per cent, the probable error of a difference is 6.6 per cent. If one considers three times the probable error of a difference as significant, then a difference in Helminthosporium reaction to be of importance must be three or more in the more susceptible lines and five or more in the resistant lines.

The numerical figure for Lion was 13.1 and for Svanhals 23.6 (Table XV). The F_3 lines varied in their reactions, some being as resistant as Svanhals while others were as susceptible as Lion. The distribution of the lines fits a normal frequency curve fairly well. From these data alone no definite conclusions can be drawn as to the number of genetic factors concerned in the production of resistance. It is of interest, however, to consider the reaction to Helminthosporium in relation to other characteristics. (See under "Linkage relations.")

INDEPENDENT INHERITANCE

BLACK VERSUS WHITE AND TWO-ROWED VERSUS SIX-ROWED.—In the cross Svanhals×Lion, the character pair 6-rowed versus 2-rowed was shown to be differentiated by a single factor. The same was shown to be the case for the character pair black versus white. Likewise, in the cross *Hordeum deficiens steudelii×Manchuria* the character pairs *deficiens* versus 6-rowed and black versus white were shown each to be differentiated by a single factor pair. The relation of the character pair 2-rowed or *deficiens* versus 6-rowed to the character pair black versus white is shown in Tables XVI and XVII. In the cross *H. deficiens×Manchuria* the factors for the characters *deficiens* and black went into the cross together and in the cross Svanhals×Lion the factor for 6-rowed went in with the factor for black. The results show the character pairs 2-rowed or *deficiens* versus 6-rowed and black versus white to be independently inherited.

TABLE XV.—*Helminthosporium reaction of F_3 lines and parent varieties in the cross Svanhals×Lion*

	Helminthosporium index						Total	Average
	12	15	18	21	24	27		
Svanhals				3	10	1	14	23.6±0.03
Lion	9	5					14	13.1±0.02
F_3 families	6	31	42	43	12	1	135	18.6

TABLE XVI.—*Distribution of F_2 plants for the characters deficiens versus six-rowed and black glume versus white. Hordeum deficiens steudelii×Manchuria*

Deficiens and intermediate black	Deficiens and intermediate white	Vulgare black	Vulgare white	Total	Year grown
211	52	60	32	355, observed	1922
200	67	67	22	356, calculated on 9:3:3:1 basis. $X^2=8.81$. $P=0.0324$.	
360	110	101	37	608, observed	1923
342	114	114	38	608, calculated on 9:3:3:1 basis. $X^2=2.60$. $P=0.4645$.	

TABLE XVII.—*Distribution of F_2 plants for the characters black glume versus white and 2-rowed versus 6-rowed; cross Svanhals×Lion*

Two-rowed and intermediate black	Two-rowed and intermediate white	Six-rowed black	Six-rowed white	Total	Year grown
651	222	221	60	1,154 observed	1922
649	216	216	72	1,153 calculated on 9 : 3 : 3 : 1 basis. X^2=2.29. P=0.52.	
280	80	88	32	480 observed	1923
270	90	90	30	480 calculated on 9 : 3 : 3 : 1 basis. X^2=1.66. P=0.6504.	

ROUGH VERSUS SMOOTH AWN AND TWO-ROWED VERSUS SIX-ROWED.—Since the data on the roughness of awn for the F_2 of the cross Svanhals×Lion grown in 1923 is close to calculated, it alone will be used in the discussion of correlated inheritance. The factors for 2-rowed and 6-rowed characters are independent of the factors for rough awn, intermediate-smooth awn, and smooth awn (Table XVIII). When the goodness of fit is calculated, the value of P is 0.2301. A deviation as large or larger than this would be expected about once in four trials.

ROUGH VERSUS SMOOTH AWN, AND BLACK VERSUS WHITE GLUMES.—The factors for black and white appear to be independent of the factors for roughness of the awn (Table XIX). A deviation as large as the one obtained would be expected in about half the trials.

LINKAGE RELATIONS

The results so far discussed show that the factors which differentiate *deficiens*, 2-rowed and 6-rowed, black versus white, rough versus smooth awn, and intermediate-smooth versus smooth

TABLE XVIII.—*Distribution of F_2 plants for the characters 2-rowed, 6-rowed, and rough awn, intermediate-smooth awn, smooth awn; cross Svanhals×Lion, 1923*

Two-rowed			Six-rowed			Total
Rough	Intermediate-smooth	Smooth	Rough	Intermediate-smooth	Smooth	
276	60	33	86	19	6	480 observed
270	67.5	22.5	90	22.5	7.5	480 calculated. X^2=6.89. P=0.2301.

TABLE XIX.—*Distribution of F_2 plants for the characters black glumes versus white and rough awn, intermediate-smooth awn, and smooth awn; cross Svanhals ×Lion, 1923*

Black glumes			White glumes			Total
Rough	Intermediate-smooth	Smooth	Rough	Intermediate-smooth	Smooth	
271	60	28	91	19	11	480 observed.
270	67.5	22.5	90	22.5	7.5	480 calculated. X^2=4.24. P=0.5178.

awn, are located in different chromosome pairs. A different relation was found to exist, however, between some of these factors and the factors for early versus late heading and the factors for resistance and susceptibility to the spot blotch disease.

TABLE XX.—*Distribution of black-glumed and white-glumed F_3 lines for date of heading; cross Svanhals × Lion, 1923*

Color of glume	Heading date			Total
	Early	Segregating	Late	
Black	7	14	3	24
White	13	26	12	51
Total	20	40	15	75

EARLY AND LATE HEADING IN RELATION TO OTHER CHARACTERS.—Fifty-one white glumed F_3 lines were grown from the cross Svanhals × Lion. Of these 51 lines, 13 bred true for early heading, 26 segregated for early versus late, and 12 bred true for late (Table XX). Of 24 lines breeding true for black glumes, 7 lines bred true for early heading, 14 segregated for early versus late, and 3 bred true for late. These results show the independence of the character pairs early versus late heading and black versus white glumes. The data for the date of heading of the rough-awn and smooth-awn F_3 families indicate here also an independence (Table XXI) although the number of lines is small in each group.

TABLE XXI.—*Distribution of rough-awn, intermediate-smooth-awn, and smooth-awn F_3 lines for date of heading; cross Svanhals × Lion, 1923*

Awn class	Heading date			Total number of families
	Early	Segregating	Late	
Rough	8	7	8	23
Intermediate-smooth	5	8	2	15
Smooth	4	16	3	23
Total	17	31	13	61

Of the 135 F_3 lines grown from the cross Svanhals × Lion, 64 were 6-rowed and 24 were homozygous 2-rowed. In the 6-rowed group, 20 lines bred true for early heading, 11 headed late, and 33 segregated (TableXXII). In the 2-rowed group the conditions are reversed; more lines bred true for late heading than for early heading. Since the 6-rowed parent in this cross is the early parent, the factors for 6-rowed and early heading went into the cross together and tend to stay together. The linkage intensity, however, is quite low.

TABLE XXII.—*Summation of the 6-rowed and 2-rowed F_3 families with respect to the characters early heading and late heading; cross Svanhals × Lion, 1923*

	Six-rowed families			
	VV EE	VV Ee	VV ee	Total
Observed	20	33	11	64
Calculated 42 per cent C. O	21.5	31.2	11.3	64

	Two-rowed families			
	vv EE	vv Ee	vv ee	Total
Observed	4	12	8	24
Calculated 42 per cent C. O	4.2	11.9	8.1	24.2

χ^2 = less than 1. P = Good fit.

In calculating the linkage intensity, genotypes are assumed for the groups as follows:

Group	F_2 genotype	F_3 breeding behavior
a	VV EE	Breeding true for early heading and 6-rowed.
b	VV Ee	Segregating for date of heading, breeding true for 6-rowed.
c	VV ee	Breeding true for late heading and 6-rowed.
c	vv EE	Breeding true for early heading and 2-rowed.
b	vv Ee	Segregating for date of heading, breeding true for 2-rowed.
a	vv ee	Breeding true for late heading and 2-rowed.

In independent inheritance, the groups *a*, *b*, and *c* are to each other as 1:2:1 or $\sqrt{\frac{a}{c}}:1$ equals 1:1, $\frac{2a}{b}:1$ equals 1:1, and $\frac{b}{2c}:1$ equals 1:1. If one combines these three terms, the formula becomes

$$\left[\left(\sqrt{\frac{a}{c}}+\frac{2a}{b}+\frac{b}{2c}\right)\Big/3\right]:1.$$

This formula, in independent inheritance, gives 1:1, or the ratio of the gametes VE to Ve and ve to vE. In the case of linkage, the formula in the bracket will equal the noncrossover gametes in relation to one crossover gamete when the factors V and E enter the cross from one parent and v of linkage with 42 per cent crossover the fit is unusually good, X^2 being less than unity (Table XXII).

This method of deriving gametic ratios and crossover percentages may prove of considerable value for those cases in which a selection is made in F_2 for noncorrelated characters. The

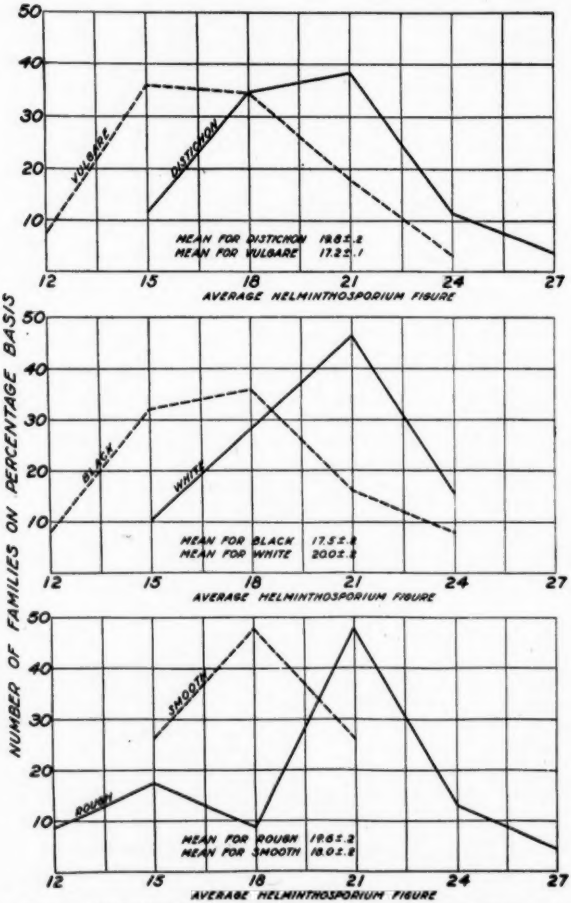


FIG. 1.—Distribution for Helminthosporium reaction of F_3 lines homozygous for other characters, cross Svanhals×Llon, 1923

and the other. As selection was made for the characters 2-rowed and 6-rowed and the number of families in the 6-rowed group is different from the number in the 2-rowed group, the formula should be applied to each group separately and the two results averaged. When the observed results are compared to calculated results on the basis fact that it has been valuable in this one case is considered sufficient justification for including it in this discussion.

REACTION TO HELMINTHOSPORIUM IN RELATION TO OTHER CHARACTERS.—Considerable variability is found in the reaction of a variety, even a pure line, to *Helminthosporium sativum*. For this

reason, resistance and susceptibility can not be considered as absolute characters, and it is impossible from the data obtained on an F_3 line to conclude whether or not the line is homozygous for resistance. The relation of resistance and susceptibility to other characters can be studied only by considering these groups in a comparative manner.

Keeping in mind that the factors for resistance to Helminthosporium went into the cross Svanhals × Lion with the factors for rough awn and white glumes, it is interesting to note that the white-glumed F_3 lines tend to be more resistant than the black lines and the rough-awned lines more resistant than the smooth-awned lines. (Fig. 1 and Tables XXIII and XXIV.)

TABLE XXIII.—*Relation of resistance and susceptibility to other characteristics in F^3 families of the cross Svanhals × Lion*

Distribution of F_3 families of—	Average Helminthosporium figure						Total	Average
	12	15	18	21	24	27		
2-rowed		3	9	10	3	1	26	19.8±0.4
6-rowed	5	24	23	12	2		66	17.2±0.2
Black	2	8	9	4	2		25	17.5±0.4
White		4	11	18	6		39	20.0±0.3
Rough	2	4	2	11	3	1	23	19.6±0.5
Smooth		6	11	6			23	18.0±0.3

The character pairs black versus white, 2-rowed versus 6-rowed, and rough awn versus smooth awn, were shown to be independent. Since resistance and susceptibility tend to be correlated with each of these three character pairs, it may be inferred that the chromosomes which carry the factors for the characters mentioned also carry factors for resistance and susceptibility to the spot blotch disease. Thus it may be said that at least three genetic factors are concerned in producing resistance of the type possessed by Svanhals. That resistance and susceptibility are due to definite genetic factors is shown by the fact that either may be combined with black or white, 2-rowed or 6-rowed, and rough or smooth.

TABLE XXV.—*Comparison of the Helminthosporium figure for the 6-rowed and 2-rowed F_3 lines in regard to the characters early and late*

	Average Helminthosporium figure						Total	Average figure for lines
	12	15	18	21	24	27		
6-rowed early	3	10	7				20	15.6±0.3
6-rowed late			2	7	1		10	20.7± .3
6-rowed segregating	2	11	15	5	1		34	17.3± .3
2-rowed early			4				4	18.0
2-rowed late		1		4	2	1	8	22.4±0.8
2-rowed segregating		2	5	4	1		12	19.0± .5

TABLE XXIV.—*The distribution of rough-awn and smooth-awn F_3 families in regard to Helminthosporium figure as influenced by other characters*

Families	2-rowed or 6-rowed	Black or white	Helminthosporium index						Total	Average
			12	15	18	21	24	27		
Rough-awn	6-rowed	Black	2	2					4	13.5
		White		2	1	2	1		6	19.0
	2-rowed	Black			1	7	2	1	11	21.8
		White				2			2	21.0
Total			2	4	2	11	3	1	23	19.6±0.5
Smooth-awn	6-rowed	Black		2	2	1			5	17.4
		White		1	3	2			6	18.5
	2-rowed	Black		3	5	2			10	17.7
		White			1	1			2	19.5
Total				6	11	6			23	18.0± .3
Intermediate smooth-awn	6-rowed	Black		3	2				5	16.2
		White			2	1			3	20.0
	2-rowed	Black	5	3	1	3			7	18.0
		White			1	3			4	20.0
Total				6	6	7			19	18.2± .4

Since the factor for the 6-rowed character is linked with the factor for early heading, the 6-rowed lines and the 2-rowed lines should be examined to see if a correlation exists for relation to Helminthosporium, irrespective of the characters early and late. When the average Helminthosporium figure for the 6-rowed early lines is compared with that for the 2-rowed early lines, a difference is found still to exist for Helminthosporium reaction (Table XXV). A difference is found also between the 6-rowed lates and the 2-rowed lates, likewise between the 6-rowed lines and the 2-rowed lines which are segregating for early versus late. If each of these groups is given equal weight, the figure for the 6-rowed lines is 17.9 ± 0.2 [6] and for the 2-rowed lines 19.6 ± 0.4, the difference being 2 ± 0.4, which is significant when considered in the light of its probable error.

The differences between the Helminthosporium figures of the early-heading and late-heading lines is greater than the difference between the figures for the 2-rowed and 6-rowed lines. This difference may be due to a physiological resistance of lateness or to a more intense linkage between the factor for earliness with the factor for susceptibility than exists between the factor for 6-rowed and the factor for susceptibility.

In considering the degree of resistance or susceptibility in relation to other characters, one is justified in the conclusion that at least three factor pairs are concerned in the production of resistance and susceptibility. One of these factor pairs is located in the chromosome pair carrying factors for 2-rowed and 6-rowed, one in the chromosome pair carrying factors for black and white glumes, and the other in the chromosome pair carrying factors for rough awn and smooth awn.

The data given here on the relation of factors for resistance to Helminthosporium and the method of locating such factors through a study of their relation to factors for other characters were presented to the Genetics Group of the American Association for the Advancement of Science (*2*). Sax (*20*), at the same meeting, gave the results of studies in which he used a similar attack on the location of size factors. As Sax (*19*) points out, the total effect of the factor or factor group can not be estimated on account of the fact that an apparent difference in such factors may be due to differences in the intensity of their linkage with those factors with which they are associated. When, however, a sufficient number of factors have been located in a particular chromosome, it may be possible to locate a size factor definitely through studying its relation to three or more other factors.

SUMMARY

Each of the cultivated barley species, *Hordeum vulgare*, *H. intermedium*, *H. distichon*, and *H. deficiens*, has 14 somatic chromosomes. According to this, 7 linkage groups are expected.

Each of the following character pairs is shown to differ by a single genetic factor: 2-rowed versus 6-rowed, *deficiens* versus 6-rowed, black versus white glumes, early heading versus late, rough awn versus smooth, and intermediate-smooth awn versus smooth.

In the cross Svanhals × Lion the character pairs black glumes versus white, 2-rowed versus 6-rowed, rough awn versus smooth awn, and intermediate-smooth awn versus smooth were shown to be independent of each other in inheritance. Black glumes versus white glumes and *deficiens* versus 6-rowed were found to be independent character pairs in the cross *H. deficiens steudelii* × *Manchuria*.

Resistance and susceptibility to *Helminthosporium sativum* are shown to be due to definite genetic factors By studying the reaction of F_3 lines to this pathogene in relation to other characters the inference is drawn that at least three factors are concerned in the production of resistance of the type possessed by Svanhals. One factor was linked with the factor for 2-rowed, one with the factor for rough awn, and one with the factor for white glumes.

The factor for early heading was found to be linked with the factor for 6-rowed. The linkage intensity was very low, the crossover value being 42 per cent. The linkage of the factor for susceptibility to Helminthosporium with the factor for earliness is much more intense than that with the factor for 6-rowed, else earliness in itself predisposes the plant to attack by the pathogene.

From the results of the crosses discussed four linkage groups have been established.

[6] The probable error of an average of averages calculated according to the formula $E=1/N \sqrt{n_1^2 e_1^2 + n_2^2 e_2^2 \cdots n_n^2 e_n^2}$, in which n is the number of lines in a group, e the probable error of the group, and N the total number of lines considered (*17*).

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APPENDIX

TABLE XXVI.—*Distribution for date of heading in F_3 families as compared with the parent varieties; cross Svanhals × Lion, 1923*

Row No.	Date of heading									Number of plants	Classed as—	Genotype of parent plant
	18	21	24	27	30	3	6	9	12			
Lion		32	11	1			1			45	Early	EE
Do		19	16	3	1					39	do	EE
Do		23	16				1			40	do	EE
Do		18	22	1						41	do	EE
Do		32	8	3						43	do	EE
Do		21	11	4	1					38	do	EE
Do		39	2	1						42	do	EE
Do		22	6	1	6					35	do	EE
Do	1	33	9			1				44	do	EE
Do		23	9	5	4					41	do	EE
Do		26	15	2						43	do	EE
Do		23	15	4				1		43	do	EE
Do		25	13	2	2					42	do	EE
Do		20	10	2	6					38	do	EE
Svanhals						8	13	11	7	39	Late	ee
Do					1	9	20	9		39	do	ee
Do						2	16	14	5	37	do	ee
Do					2	19	19	2	1	43	do	ee
Do						6	20	5	5	36	do	ee
Do						6	17	10	4	37	do	ee
Do					1	14	25	1		41	do	ee
Do						3	19	6	6	34	do	ee
Do						15	16	7	7	45	do	ee
Do						1	20	10	4	35	do	ee
Do					1	11	22	3	2	39	do	ee
Do						3	22	9	1	35	do	ee
Do					1	15	14	7	1	38	do	ee
Do						3	25	6	3	37	do	ee
3	8	24	3	1	1		4	1		42	Segregating	Ee
4		29	14	1	2					46	Early	EE
5	1	21	7		1		3			33	Segregating	Ee
6	15	12	15							42	Early	EE
7						1	12	10	4	27	Late	ee
8							19	11	3	33	do	ee
9						1	23	6	5	35	do	ee
10	1	16	13	2			5	4	1	42	Segregating	Ee
11	23	9	5							37	Early	EE
12		17	11	2			1	4	4	39	Segregating	Ee
15		6	18	1	2	1	1	2	4	35	do	Ee
16		16	3	1	3	7	3	3		36	do	Ee
17		13	9	3	2	1	6	4	2	40	do	Ee
18		10	8	3	1	2	3	1	3	31	do	Ee
19	1	36	5	3						45	Early	EE
20	2	39	1							42	do	EE
21		18	5	2	3	3	4	3	4	42	Segregating	Ee
22		9	13	2	2		1	3	3	33	do	Ee
23						1	19	12	2	34	Late	ee
24		18	7	4			2	1	3	35	Segregating	Ee
27		33	5	2	1					41	Early	EE
28							22	12	2	36	Late	ee
29	3	20	3	1		1	2	1		31	Segregating	Ee
30						6	15	11	2	34	Late	ee
31	1	14	15	2			1	3		36	Segregating	Ee
32	1	8	15	1	1		5	5		36	do	Ee
33		35	6							41	Early	EE
34	9	28	2							39	do	EE
35		14	11	3		3	5	2	2	40	Segregating	Ee
36	4	15	6	2	1	3	6	2		39	do	Ee
39		22	12	1	1					36	Early	EE
40	2	28	4			2	1	1		38	Segregating	Ee
41	11	27	1							39	Early	EE
42	5	30	2							37	do	EE
43	1	17	10	1			6	2	1	38	Segregating	Ee
44	1	20	8	1	2		5			37	do	Ee
45						1	14	15	8	38	Late	ee
46	3	31	5							39	Early	EE
47						1	5	15	8	29	Late	ee
48	10	27	1	1						39	Early	EE
51	7	21	4		1		1	1	2	37	Segregating	Ee
52	2	20	7		1		1	1		32	Early	EE
53	4	17		1		1	11			34	Segregating	Ee
54		20	2	2		2	6	1	1	34	do	Ee
55						2	14	7	10	33	Late	ee
56	4	15	4	1			1	2		27	Segregating	Ee
57		28	5	1		2	1	1	2	40	do	Ee
58	3	17	9			3	7		1	40	do	Ee
59		24	7	1	1	3	1			37	do	Ee
60	4	32	2		1					39	Early	EE
63		15	6	4		1	4	4	2	36	Segregating	Ee
64	2	25	3	2			3			35	do	Ee
65	4	18	4	2	1	1		2		32	do	Ee

TABLE XXVI.—*Distribution for date of heading in F_3 families as compared with the parent varieties; cross Svanhals × Lion, 1923*—Continued

Row No.	Date of heading									Number of plants	Classed as—	Genotype of parent plant
	18	21	24	27	30	3	6	9	12			
66		37	3	1						41	Early	EE
67						5	20	11	4	40	Late	ee
68	2	24	3	1	1		5	3		39	Segregating	Ee
69		21	6	2		1	1	1		32	Early	EE
70		11	10	2	2	1	5	3		34	Segregating	Ee
71		15	5	1	4	2	1	1	1	30	do	Ee
72		14	6	4	1	1	1		2	29	do	Ee
73	4	19	1		2	3			1	30	do	Ee
76	1	40	1							42	Early	EE
77	4	26	3		2	1	2	1		41	Segregating	Ee
78		1				1	19	9	1	31	Late	ee
79	3	33		4						40	Early	EE
80		19	7			1	2	1	3	33	Segregating	Ee
81	3	18	4	2		2	7	1		37	do	Ee
82	1	17	10		1	1	2	4		36	do	Ee
83	3	33	4	1						41	Early	EE
84		22	6	2			2	2	3	37	Segregating	Ee
87	3	23	3		1	2	4	2	1	39	do	Ee
88	7	24	2			2	2	4		41	do	Ee
89	1	25	6			1	6	2		41	do	Ee
90					1	16	11	4	1	33	Late	ee
91					1	16	14	3	1	35	do	ee
92	16	23	2	1	1					45	Early	EE
93		23	7		1	4	4	2	1	42	Segregating	Ee
94						10	14	10	3	37	Late	ee
95						7	26	5	1	39	do	ee
96						5	22	8	2	37	do	ee
99	2	25	3	1			4		1	36	Segregating	Ee
100	2	27	3			4	2			38	do	Ee
101			1		2	5	14	10	5	37	Late	ee
102	5	21	2	2	1	4	3	1		39	Segregating	Ee
103	21	11	6							38	Early	EE
104	9	31								40	do	EE
105	12	29		1						42	do	EE
106	6	21		1		2	2	2	2	36	Segregating	Ee
107	2	27	1		2	6	2	2		42	do	Ee
108	5	31								36	Early	EE
111	18	23	2	1						44	do	EE
112	1	40	1		1					43	do	EE
113	3	25	2	4	1			1	2	38	Segregating	Ee
114						1	18	10	5	44	Late	ee
115				1	1	16	11	2	3	34	do	ee
116						1	5	15	12	33	do	ee
117	16	16	1							33	Early	EE
118						6	20	7	4	37	Late	ee
119	10	26	3		1		1			41	Early	EE
120	8	19	6		2	3	1			39	Segregating	Ee
123	11	14	4			3	3			35	do	Ee
124	5	25	4	1						35	Early	EE
125	2	25	1	2		3	6			39	Segregating	Ee
126					2	15	17	3	1	38	Late	ee
127	2	25		1			4	7	1	40	Segregating	Ee
128						12	20	6	2	40	Late	ee
129	4	15	1	1	1	1	6	3	2	34	Segregating	Ee
130	5	20	3	3		5	1			37	do	Ee
131							25	10	2	37	Late	ee
132						3	22	8	3	36	do	ee
135					2	13	15	8	1	39	do	ee
136	7	23	1	2			2	2		37	Segregating	Ee
137	2	22	1	1	1	4	6	3		40	do	Ee
138	12	14	1	1	2	7	4	1		42	do	Ee
139	16	18	1	2	3	2		2		44	do	Ee
140	3	24	2			1	1		1	32	Early	EE
141						6	19	8	1	34	Late	ee
142	2	31	1							34	Early	EE
143		14	6	1	4	1	4	4		34	Segregating	Ee
144	2	26	3				4	1	1	37	do	Ee
147	4	23	4			3	4			38	do	Ee
148		25	7				3	3	2	40	do	Ee
149	6	15	1			1	3	1		27	do	Ee
150		20	6		2			1	2	31	do	Ee
151	1	21	6	2			3	3	2	38	do	Ee
152		23	4	1	1	1	2			32	do	Ee
153	1	25	2			2	6	3	1	40	do	Ee
154		1				1	23	13	3	41	Late	ee
155	1	24	4				2	2	6	39	Segregating	Ee
156							15	12	6	33	Late	ee
159	3	15	5	2	1	1	4	1	2	34	Segregating	Ee
160							11	15	12	38	Late	ee
161	17	19	1							37	Early	EE
162	6	17	3	1	2	1	4	1		35	Segregating	Ee
163	1	16	7	3	2		1	1	2	33	do	Ee

THE RELATION OF SULPHUR TO ALFALFA PRODUCTION [1]

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INTRODUCTION

That sulphur is one of the essential elements for the proper growth and development of plants is a well-known fact. Being a constituent of most proteins which are closely associated with living matter, it probably is connected with physiological processes in the formation of compounds which do not contain sulphur. If this be true, it is apparent that sufficient quantities of sulphur must be supplied to plants in order to produce normal maximum growth.

In recent years the importance of sulphur in crop production has been given greater consideration than formerly. Probably this is due to the early and erroneous methods of analysis which indicated that crops removed very small amounts of this element from the soil. Hence it was thought that if crop residues were returned, most soils contained enough sulphur to produce maximum yields indefinitely. However, improved methods of analysis have shown that considerably more sulphur is removed by plants than was formerly thought to be the case and that in some instances it may be the limiting factor in crop production.

In field experiments sulphur deficiency has been observed most frequently with legumes. Since alfalfa is one of the most important legumes, it was deemed desirable to investigate the possibility of increasing the yields of this crop by sulphur fertilization. This paper reports 10 results of a single experiment conducted for one season.

REVIEW OF LITERATURE

Reimer and Tartar (*13*) [3] in Oregon found that the yields of alfalfa and red clover can be greatly increased by the use of sulphur-containing fertilizers. The soils on which these experiments were conducted contained only limited amounts of sulphur. Superphosphate and gypsum were found to increase the yield of alfalfa and produced a darker green color, while rock phosphate had little or no effect whatever. Other sulphur-containing fertilizers increased the yields from 50 to 1,000 per cent, larger increases being obtained in some cases than in others where the same amount of sulphur was applied. These differences were attributed to differences in depth and physical condition of the soil. The application of fertilizers containing nitrogen, phosphorus, and potassium had little or no effect on alfalfa. The same was true regarding the application of lime. This was taken to indicate that any benefits derived from sulphur-containing fertilizers could not be due to their effect upon liberating phosphorus, potassium, or lime in the soil, or upon nitrification. The root system and the number of nodules was also materially increased by the addition of sulphur. Plants receiving sulphur contained more nitrogen and more protein than those from the control plots.

Fellers (*5*) secured a material increase in the yield of total dry matter and seed of soy beans by small applications of acid phosphate on well limed soils. Small applications seemed to be as effective as larger amounts. It was observed also that acid phosphate applied on limed soil increased the nodule development and content of oil. Protein formation was benefited on both limed and unlimed soils. Land plaster produced but little effect unless applied in large amounts, in which case the oil content of the seed and the growth of nodules were stimulated. Applications of elemental sulphur in small amounts, not over 100 pounds

[1] Received for publication July 29, 1924; issued July, 1925. Contribution No. 152, Department of Agronomy, Kansas Agricultural Experiment Station. Although this paper reports the results of but a single experiment conducted for one season, it is thought desirable to publish the results for the benefit of other workers, since there is no immediate prospect of continuing the work.

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[3] Reference is made by number (italic) to "Literature cited," p. 947.

per acre, produced an increase in yield, but larger amounts seemed to injure the plants. The protein content was increased by moderate applications of sulphur and decreased by large amounts. Duley (*2*) has also shown that gypsum and sulphur greatly increase nodule formation on red clover in certain Missouri soils.

Hall (*6*) found that the percentage of total sulphur in alfalfa hay from various parts of the United States varied to a considerable degree and that the fields producing the heaviest yields contained the highest percentages of sulphur. The amount of sulphur removed by average crops was found to be from 37 to 90 pounds per acre, which seems to be in excess of the amount returned to the soil by rain.

Eaton (*3*) pointed out that most of the sulphur in the soil is in the organic form and that there is a general correlation between the sulphur and organic matter content, soils high in organic matter having in general a high sulphur content. It was found that the application of large amounts of gypsum to red clover produced plants containing more nitrates, protein, and sulphates than where small amounts were added. Also flowers of sulphur and sodium sulphate containing the same amount of sulphur as 100 pounds of gypsum per acre and gypsum at the rate of 500 pounds per acre greatly increased the yield of sweet corn. Larger applications of flowers of sulphur and sodium sulphate gave no increase.

In regard to beans and peas, Hart and Tottingham (*7*) found that sulphates were beneficial in the production of seed and of hay. Calcium sulphate was found to be superior to sodium sulphate; however, both greatly increased root development and likewise produced more hay. Elemental sulphur depressed the development of the legumes studied except for an apparent increase in root development in clover. Miller (*10*) found an increase in the nitrogen content and root development of clover by the application of sulphates.

Shedd (*15*) secured an increased yield of soy beans in almost every test from the application of sulphates or sulphur. Sulphatic fertilizers increased the sulphur content of soy beans, but not necessarily the protein content, as would be expected, since sulphur is a constituent of protein. In a number of cases the per cent of protein increased as the sulphur content increased, but not in the same proportion. Ammonium sulphate was found to increase the protein content of soy beans to a marked degree.

Olson and St. John (*11*) found that alfalfa treated with sulphur-containing fertilizers produced two or more times the quantities of hay on certain Oregon soils than was obtained on land untreated. Alfalfa treated with sulphur contained more protein than where no sulphur was applied and the plants had a darker green color which was evident in the cured hay.

Pitz (*12*) concluded that calcium sulphate greatly increased root development in clover grown in artificial media, but concentrations as high as 0.1 per cent retarded root growth. Elemental sulphur gave a slight increase in yield of red clover on Miami silt loam, but there was no difference in size or number of nodules on the roots. Large amounts of elemental sulphur decreased the total number of bacteria growing on agar plates, but produced an increase in ammonification with concentrations of 0.05 per cent. This was accompanied by a decrease in nitrate formation, which was thought to be due to the acidity produced.

Erdman (*4*) obtained an increased yield of alfalfa hay at each cutting with gypsum, but the effect was more pronounced on the first cutting than on subsequent cuttings. Apparently 200-pound applications were better than 500-pound applications. Gypsum did not affect the nitrogen content of the hay. He also found that gypsum was favorable to red clover in several instances and that it had practically no effect on the crude protein content with or without lime. It was found that gypsum applied in ordinary amounts had no effect upon the types of bacteria instrumental in breaking down organic matter nor was there any apparent effect upon the ammonifiers or nitrifiers. This is practically in accord with results reported by Lipman (*9*) who states that from 200 to 500 pounds of sulphur per acre did not materially increase the lime requirement, but heavier applications did increase the lime requirement.

PLAN OF INVESTIGATION

In order to determine the effect of sulphur on alfalfa production a greenhouse experiment was planned consisting of 12 treatments of five pots each. Each pot contained 12.24 kg. of the surface 7 inches of Oswego silt loam, a black, productive residual soil derived from weathered shale. Soil samples were taken at the beginning of the experiment for the purpose of

determining the content of moisture, nitrogen, phosphorus, and sulphur. The method of treatment for the various series was as follows:

No. 1, acid phosphate 400 lbs. per acre.
No. 2, acid phosphate 400 lbs. and sulphur 384 lbs. per acre.
No. 3, lime as calcium oxide 4,000 lbs. per acre.
No. 4, sulphur 384 lbs. and lime 4,000 lbs. per acre.
No. 5, sulphur 384 lbs. per acre.
No. 6, 542 lbs. of N–P–K as 2–12–2, and lime 4,000 lbs. per acre.
No. 7, 542 lbs. of N–P–K as 2–12–2, 384 lbs. of sulphur and 4,000 lbs. of lime.
No. 8, manure 3,000 lbs. per acre.
No. 9, manure 3,000 lbs. and sulphur 384 lbs. per acre.
No. 10, manure 3,000 lbs., sulphur 384 lbs., and lime 4,000 lbs. per acre.
No. 11, control.
No. 12, inoculated sulphur 384 lbs. per acre.

The sources of fertilizing elements in the complete fertilizer were nitrogen from $NaNO_3$, phosphorus from $CaH_4(PO_4)_2$, and potassium from KCl. The different materials were added to the pots and thoroughly incorporated with the soil before bringing the pots up to the optimum water condition. The alfalfa was then planted, November 22, 1922. Because of a delay in getting a supply of inoculated sulphur, series No. 12 was not seeded until December 6, 1922. Each pot was weighed twice per week and enough distilled water added to bring it up to weight. When the plants were about half grown they became infested with red spider. The only effective way of controlling this pest is a rather strong spray of cold water from a hose. The spraying has to be done every day the sun shines and, as a consequence, some water other than distilled water was applied in this way. The plants were allowed to grow until the majority were in full bloom when they were harvested, May 8, 1923. After recording the green weights, the samples were placed in a steam drying oven and the oven-dry weights determined.

The method used in determining the content of nitrogen, phosphorus, and potassium was the same as stated in the manual of the Association of Official Agricultural Chemists, revised in 1919 (*1*). The magnesium nitrate method, as described by Swanson and Latshaw (*17*) was used in determining the sulphur content of the soil. The magnesium nitrate method, as described by Latshaw (*8*), was used in determining the sulphur in the roots and tops of the plants. The colorimetric method, as given by Schreiner and Failyer (*14, p. 49*), was used in determining the soluble sulphates of the soil.

EXPERIMENTAL RESULTS

The composition of the Oswego silt loam at the beginning of the experiment is as follows: Surface soil—total moisture, 19.31 per cent; nitrogen, 0.228 per cent; phosphorus, 0.061 per cent; potassium, 2.119 per cent; total sulphur, 0.065 per cent; P_H value, 7.016 per cent; and soluble sulphates, 34.6 parts per million. In general these figures agree rather favorably with results of Swanson and Miller (*16*) but the potassium and sulphur content is somewhat higher than that reported by these investigators.

The mechanical analysis of Oswego silt loam as reported by the Bureau of Soils, United States Department of Agriculture, is as follows: Surface soil—fine gravel, 0.0 per cent; coarse sand, 0.6 per cent; medium sand, 0.3 per cent; fine sand, 1.5 per cent; very fine sand, 6.9 per cent; silt, 71.8 per cent; and clay, 17.8 per cent.

The daily minimum and maximum temperatures for the period of the experiment are shown in Table I. No records for the week ending December 30, 1922, are available.

The yields for the first and second cuttings of each treatment are given in Table II, in which it will be observed that the treatments which have given the greatest returns are lime, sulphur lime, and N–P–K with sulphur and lime (pl. 1). The difference in mean yield between the series named above in the respective order and the control is 2.0, 3.0, and 3.9 gm. per pot. These treatments, however, do not give distinctly significant increases when the probable error calculated by Bessel's formula is considered.

The weight of roots for the various treatments are given in Table III. The probable errors calculated by Bessell's formula are given for the yields but the numbers in which they are based are small and probably they should be used with caution in interpreting the results.

Acid phosphate and sulphur, sulphur, N–P–K with lime, manure, and inoculated sulphur are treatments resulting in decreases in yield of alfalfa (pl. 2). Here again, the decreases are not significant on the basis of the probable error as calculated by Bessel's formula.

Considering root growth, as shown in Table III, there are not any significant increases in the effect of the various treatments, but three significant decreases: Acid phosphate;

manure and sulphur; manure, sulphur, and lime, all produced decreases in root development greater than three times the probable error of the difference. A careful examination of the roots was made for nodules but no differences in the size or number corresponding to differences in treatment were observed.

Plates 1 and 2 portray the differences in top growth for the first and second cuttings. For the first cutting there was a noticeably better growth of alfalfa in the limed and sulphur-with-lime treatments. This difference was not borne out in the second cutting, all treatments producing approximately the same growth response.

TABLE I.—*Minimum and maximum daily temperature*

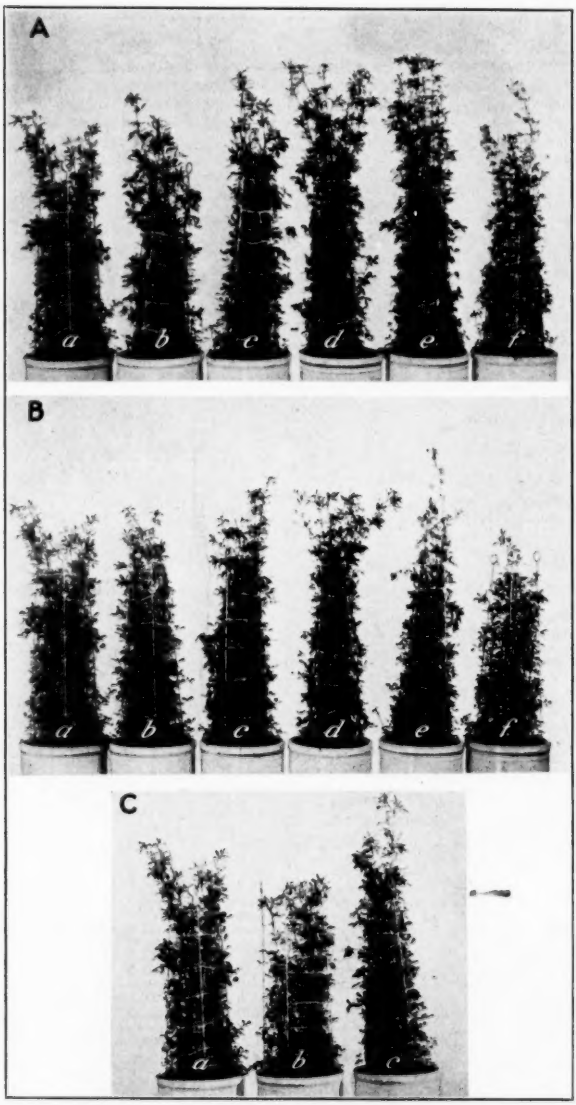
Week ending—	Sunday		Monday		Tuesday		Wednesday		Thursday		Friday		Saturday	
	Min.	Max.	Min.	Max.	Min.	Max.	Min.	Max.	Min.	Max.	Min.	Max.	Min.	Max.
	°F.	°F.	°F.	°F.	°F.	°F.	°F.	°F.	°F.	°F.	°F.	°F.	°F.	°F.
Nov. 25	59	77	64	73	63	80	58	85	58	80	51	77	52	91
Dec. 2	53	83	56	83	60	78	62	87	52	82	57	90	55	80
Dec. 9	50	73	58	81	49	85	60	76	57	73	57	77	53	76
Dec. 16	57	72	59	72	56	88	50	77	58	78	58	81	61	78
Dec. 23	57	81	61	90	61	82	65	87	60	82	63	81	63	85
Jan. 6	60	93	58	78	50	81	60	85	74	83	60	83	63	84
Jan. 13	65	94	67	78	65	81	68	86	65	83	67	86	60	83
Jan. 20	64	80	54	86	66	87	69	82	60	76	66	82	62	88
Jan. 26	56	80	62	85	62	78	63	85	67	81	61	82	69	86
Feb. 2	71	87	68	88	68	76	63	73	64	91	66	88	61	88
Feb. 10	70	95	66	86	65	87	65	80	69	88	58	85	58	80
Feb. 17	70	93	69	90	57	86	64	80	64	88	63	86	60	83
Feb. 24	74	87	68	81	65	85	69	90	55	88	67	90	71	88
Mar. 3	70	92	64	87	63	95	59	84	70	95	70	92	57	85
Mar. 10	65	86	65	81	60	95	65	88	63	90	62	92	70	92
Mar. 17	67	76	65	82	61	95	61	77	68	80	63	92	65	92
Mar. 24	52	88	66	85	71	88	62	95	62	80	63	88	65	97
Mar. 31	61	88	60	95	61	92	65	90	61	95	60	75	66	95
Apr. 7	57	90	67	78	63	80	67	72	68	80	70	81	61	81
Apr. 14	72	85	66	77	65	80	67	81	68	78	68	85	63	76
Apr. 21	65	81	63	82	70	86	68	85	75	83	68	77	65	79
Apr. 28	65	75	67	86	62	85	67	75	64	78	65	75	65	82
May 5	63	88	65	85	70	80	65	88	65	87	65	83	60	90
May 12	60	91	67	84	57	83	57	83	63	94	70	87	60	89

TABLE II.—*Yields of different treatments for first and second cuttings*

Pot No.	Treatment	Dry weight			Difference in yield compared with control	Ratio $\frac{D}{Ed}$
		First cutting	Second cutting	Total		
		Gm.	Gm.	Gm.	Gm.	
1	Acid phosphate	40.0	47.5	87.5		
2	do	42.5	44.5	87.0		
3	do	42.5	43.0	85.5		
4	do	36.5	42.0	78.5		
5	do	47.5	37.0	84.5		
	Average			84.6 ±1.085	−0.4±3.32	0.12
6	Acid phosphate and sulphur	43.0	45.0	88.0		
7	do	34.5	42.0	76.5		
8	do	31.0	39.5	70.5		
9	do	29.0	40.5	69.5		
10	do	36.5	40.5	77.0		
	Average			76.3 ±2.273	−8.7±3.87	2.24
11	Lime	39.5	44.5	84.0		
12	do	44.5	47.0	91.5		
13	do	46.5	46.0	92.5		
14	do	34.3	42.5	77.0		
15	do	47.5	42.5	90.0		
	Average			87.0 ±1.956	−2.0±3.70	0.54

TABLE II.—*Yields of different treatments for first and second cuttings*—Continued

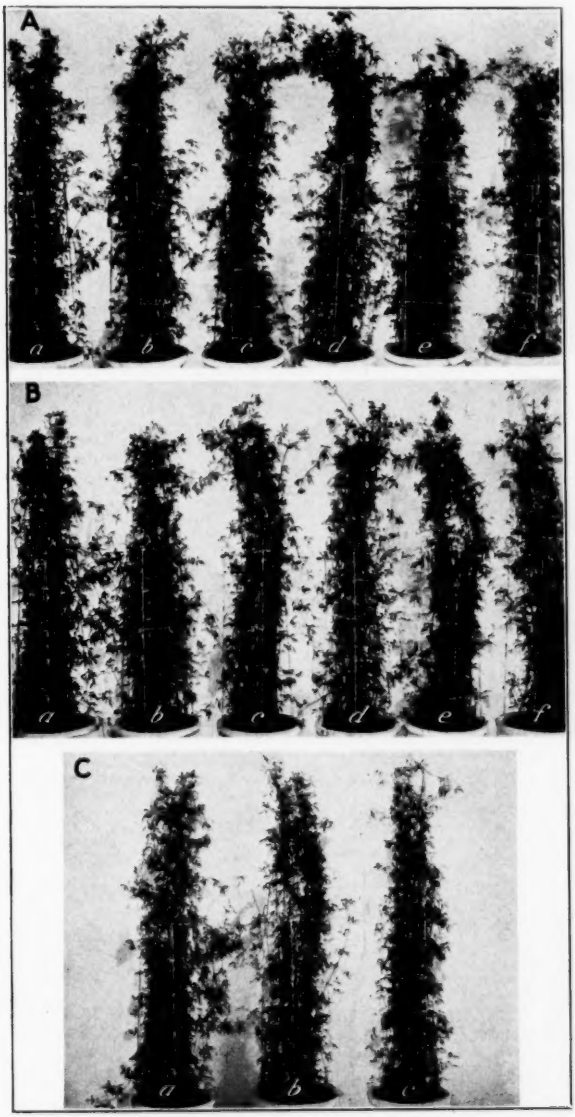
Pot No.	Treatment	Dry weight			Difference in yield compared with control	Ratio $\frac{D}{Ed}$
		First cutting	Second cutting	Total		
		Gm.	*Gm.*	*Gm.*	*Gm.*	
16	Sulphur and lime	41.5	45.5	87.0		
17	do	39.0	46.8	85.5		
18	do	48.0	49.5	97.5		
19	do	55.5	45.0	100.5		
20	do	30.5	39.0	69.5		
	Average			88.0 ±3.682	−3.0±3.68	0.81
21	Sulphur	42.5	35.5	78.0		
22	do	42.5	41.0	83.5		
23	do	34.0	41.0	75.0		
24	do	32.0	45.5	77.5		
25	do	31.5	44.5	76.0		
	Average			78.0 ±0.694	−7.0±3.20	2.18
26	N–P–K and lime	36.5	47.0	83.5		
27	do	38.0	45.5	83.5		
28	do	37.0	39.5	76.5		
29	do	39.5	47.0	86.5		
30	do	32.5	47.5	80.0		
	Average			82.0 ±1.36	−3.0±3.42	0.87
31	N–P–K, sulphur, and lime	39.5	54.0	93.5		
32	do	47.5	49.5	97.0		
33	do	46.5	44.5	91.0		
34	do	34.0	45.5	79.5		
35	do	38.0	45.5	83.5		
	Average			88.9 ±2.178	−3.9±3.82	1.02
36	Manure	35.0	43.0	78.0		
37	do	33.5	40.0	73.5		
38	do	36.0	44.0	80.0		
39	do	34.5	44.0	78.5		
40	do	47.0	41.5	88.5		
	Average			79.7 ±1.666	−5.7±2.77	2.05
41	Manure and sulphur	41.0	42.5	83.5		
42	do	48.0	46.5	94.5		
43	do	41.5	46.0	87.5		
44	do	40.5	42.5	83.0		
45	do	38.5	41.5	80.0		
	Average			85.7 ±2.50	− 7±3.24	0.21
46	Manure, sulphur, lime	35.5	33.5	69.0		
47	do	34.0	43.0	77.0		
48	do	38.0	46.0	84.0		
49	do	49.5	45.0	94.5		
50	do	31.5	58.5	90.0		
	Average			82.9 ±2.994	−2.1±4.22	0.49
51	No treatment	35.0	33.5	68.5		
52	do	36.5	43.0	79.5		
53	do	38.0	46.0	84.0		
54	do	33.5	45.0	78.5		
55	do	36.0	58.5	94.5		
	Average			81.0 ±3.143		
56	Inoculated sulphur	32.5	39.5	72.0		
57	do	34.5	40.0	74.5		
58	do	33.5	39.5	73.0		
59	do	38.5	42.5	81.0		
60	do	38.0	42.5	80.5		
	Average			76.2 ±1.281	−8.8±3.38	2.60



A.—First cutting of alfalfa showing growth of plants under various treatments; *a*, Control; *b*, Acid phosphate; *c*, Sulphur; *d*, Lime; *e*, Sulphur and lime; *f*, Manure

B.—First cutting of alfalfa, showing character of growth; *a*, Control; *b*, N-P-K plus lime; *c*, N-P-K plus lime and sulphur; *d*, Manure and sulphur; *e*, Manure plus sulphur and lime; *f*, Acid phosphate and sulphur

C.—First cutting of alfalfa showing the growth of plants; *a*, Control; *b*, Inoculated sulphur; *c*, Sulphur



A.—Second cutting of alfalfa at time of harvest; *a*, Control; *b*, Acid phosphate; *c*, Sulphur; *d*, Lime; *e*, Sulphur and lime; *f*, Manure

B.—Second cutting of alfalfa; *a*, Control; *b*, N–P–K plus lime; *c*, N–P–K plus lime and sulphur; *d*, Manure and sulphur; *e*, Manure plus sulphur and lime; *f*, Acid phosphate plus sulphur

C.—Second cutting of alfalfa showing growth of plants; *a*, Control; *b*, Inoculated sulphur; *c*, Sulphur

TABLE III.—*Weight of roots for various treatments*

Pot No.	Treatment	Dry weight	Difference compared with control	Ratio $\frac{D}{Ed}$
		Gram	*Gram*	
1	Acid phosphate	26.5		
2	do	22.0		
3	do	27.5		
4	do	24.0		
5	do	17.5		
	Average	23.5 ±1.10	−7.7±1.42	5.4
6	Acid phosphate and sulphur	32.0		
7	do	25.0		
8	do	40.0		
9	do	33.5		
10	do	37.5		
	Average	33.6±0.19	−2.4±0.87	2.7
11	Lime	29.5		
12	do	35.0		
13	do	27.0		
14	do	26.5		
15	do	25.0		
	Average	28.6±1.18	−2.6±1.45	1.7
16	Sulphur and lime	25.0		
17	do	39.0		
18	do	36.0		
19	do	23.0		
20	do	15.5		
	Average	27.7±3.07	−3.5±3.1	1.1
21	Sulphur	14.5		
22	do	27.0		
23	do	15.5		
24	do	36.0		
25	do	36.0		
	Average	25.8±2.79	−5.4±2.93	1.8
26	N-P-K and lime	28.5		
27	do	33.5		
28	do	21.0		
29	do	32.5		
30	do	27.5		
	Average	30.6±2.41	−0.6±2.55	0.2
31	N-P-K, sulphur, and lime	31.5		
32	do	27.0		
33	do	25.0		
34	do	26.5		
35	do	23.5		
	Average	26.7±0.903	−4.5±1.23	3.65
36	Manure	37.5		
37	do	38.0		
38	do	28.5		
39	do	30.0		
40	do	21.0		
	Average	31.0±2.117	−0.2±2.27	0.08
41	Manure and sulphur	24.5		
42	do	25.0		
43	do	32.5		
44	do	28.0		
45	do	16.5		
	Average	25.3±1.767	−5.9±1.95	3.02
46	Manure, sulphur, and lime	13.0		
47	do	20.5		
48	do	23.0		
49	do	26.5		
50	do	32.0		
	Average	23.0±2.077	−8.2±2.23	3.67

TABLE III.—*Weight of roots for various treatments*—Continued

Pot No.	Treatment	Dry weight	Difference compared with control	Ratio $\frac{D}{Ed}$
		Gram	*Gram*	
51	No treatment	30.5		
52	do	34.5		
53	do	30.0		
54	do	33.5		
55	do	27.5		
	Average	31.2±0.849		
56	Inoculated sulphur	28.5		
57	do	26.0		
58	do	32.5		
59	do	35.5		
60	do	25.0		
	Average	29.5±1.32	−1.7±1.57	1.08

Chemical determinations were made for the acidity of the soil, soluble sulphates in the soil, and for sulphur and nitrogen in the plants. These data are presented in Table IV with a summary of the yields and probable errors. This table shows that the nearest approach to a significant difference in the results of the various treatments is in the case of acid phosphate and sulphur and the inoculated sulphur (pl. 3). Both of these treatments caused decreases in yield.

The P_H values of the soil in the respective pots give a higher hydrogen-ion content for the inoculated sulphur which may account for its effect in causing a decrease in yield. The soluble sulphates present in the soil are low for the acid phosphate-sulphur treatment compared with all others; however the percentage of sulphur in the tops equaled that present with other treatments except in the roots.

The nitrogen analyses would indicate that about the same amount of protein was formed with the respective treatments.

TABLE IV.—*Summarized data comparing effect of treatment with chemical determinations*

Treatment	Difference in yield compared with control. Total both cuttings	Difference necessary to be significant (3.2 × P. E.)	P_H value of soil after—		Soluble sulphates in soil	Sulphur in tops, first cutting	Sulphur in roots	Nitrogen in tops, first cutting
			First crop	Second crop				
					P. p. m.	*Per cent*	*Per cent*	*Per cent*
Acid phosphate	−0.4	10.6	7.827	7.151	40.3	0.4375	0.4237	3.13
Acid phosphate and sulphur	−8.7	12.38	7.151	6.678	21.1	.4877	.3157	3.39
Lime	−2.0	11.8	8.402	8.368	36.8	.4295	.4023	3.27
Sulphur and lime	−3.0	11.7	8.368	8.639	67.3	.4238	.3799	3.13
Sulphur	−7.0	10.2	6.881	7.084	43.2	.4361	.4244	3.18
N–P–K and lime	−3.0	10.9	8.233	7.861	45.0	.4591	.3832	3.43
N–P–K, sulphur, and lime	3.9	12.2	8.233	8.639	73.5	.4714	.4378	3.33
Manure	−5.7	8.8	5.664	6.407	46.8	.4330	.3957	3.33
Manure and sulphur	−0.7	10.3	8.997	7.151	47.8	.4359	.4372	3.10
Manure, sulphur, and lime	−2.1	13.5	8.436	8.436	49.9	.4246	.4462	3.08
No treatment			6.441	6.137	54.3	.4451	.4045	3.35
Inoculated sulphur	−8.8	10.8	4.514	4.311	76.4	.4203	.3967	3.23

An average-sized root system of alfalfa plants removed from the soil immediately after harvesting the second crop

SUMMARY

The data presented in these investigations show that the type of soil used in these experiments contained as much of the common fertilizing elements as the average Kansas soil and the sulphur content to be a little higher than the average.

Sulphur applied to alfalfa has given no marked increase in yield or root development. This means that sulphur is not the limiting factor in alfalfa production on the type of soil used in this experiment.

In general, the acidity of the soil was increased by the sulphur, evidence that it should be supplemented with lime.

The nitrogen content of the tops, and the sulphur content of roots and tops were not influenced by the sulphur applied.

There was not a material increase in root development nor any increase in number or size of nodules on the roots as a result of sulphur applications.

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CARBON TRICHLORIDE AS AN ANTHELMINTIC, AND THE RELATION OF ITS SOLUBILITY TO ANTHELMINTIC EFFICACY[1]

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INTRODUCTION

On the basis of a very careful and extensive study of the correlation of the chemical composition and anthelmintic efficacy of a number of drugs, Caius and Mhaskar (*3*)[2] arrived at the conclusion that since chloroform showed considerable anthelmintic efficacy against hookworms, some related compound with a higher halogen content might prove even more effective, the anthelmintic efficacy in the case of chloroform being correlated with its chlorine content. By a shorter and less exhaustive line of reasoning, Hall (*6*) derived the same conclusion from substantially the same premises and reported that carbon tetrachloride was such an anthelmintic, his paper appearing the same month that Caius and Mhaskar predicted the likelihood of a compound related to chloroform being effective against hookworms. Since chloroform ($CHCl_3$) and carbon tetrachloride (CCl_4) show considerable efficacy against hookworms, it seems desirable that other related compounds should be investigated. In a study of miscellaneous anthelmintics, Hall and Shillinger (*7*) have tested ethylene dichloride ($C_2H_4Cl_2$), and find that it has some efficacy against hookworms, but less than chloroform and carbon tetrachloride. Recently Hall and Shillinger (*8*) have reported that tetrachlorethylene (C_2Cl_4) is apparently even more effective than carbon tetrachloride in removing hookworms, their paper covering experiments carried out subsequent to those reported in this paper and continuing the same line of investigation. The investigation reported in this paper pertains to carbon trichloride or hexachlorethane (C_2Cl_6).

Carbon trichloride occurs in the form of colorless crystals which are insoluble in water, but soluble in alcohol, ether, carbon tetrachloride, and all oils. Its insolubility in water is a conspicuous property. When 1 gram was added to 2,000 c. c. of water there was no apparent diminution in the amount of it after two months, so little went into solution. The apparent solubility was decidedly less than 1 in 10,000. Its odor is suggestive of camphor or turpentine. On theoretical grounds, certain possibilities as regards anthelmintic action might be predicted from the chemical composition and physical nature of this substance. From its atomic structure one might conclude that it would be less effective than carbon tetrachloride, since its proportion of chlorine to carbon is 3 : 1 instead of 4 : 1. However, since the molecule actually contains more chlorine (6 atoms) than does carbon tetrachloride (4 atoms), it might prove actually more effective. Actual test throws no light on this subject of chlorine content versus chlorine concentration, for the decisive factor in connection with its internal action is the physical factor of solubility.

Anthelmintics have long been regarded as relatively insoluble, more or less toxic, substances, which, by virtue of their relative insolubility, would remain in the digestive tract to a great extent and poison the worms present, the host meanwhile absorbing very little of the poison and the drug being swept out of the digestive tract by means of a purge as soon as feasible. However, Hall (*5*) has noted as a principle of anthelmintic medication that "anthelmintics of the supposedly insoluble type are not as insoluble as they are commonly supposed to be," and cites the findings of Seidell in regard to thymol, to the effect that "of the thymol administered, from one-half to two-thirds is apparently destroyed or temporarily fixed in the body." Thymol, we may recall, is soluble about 1 part in 1,100 parts of water at 25° C. Carbon tetrachloride is soluble 1 part in 1,250 parts of water at 25° C.

As carbon tetrachloride appears to be about the least water-soluble of the anthelmintics in use for hookworms,

[1] Received for publication July 31, 1924; issued July, 1925.
[2] Reference is made by number (italic) to "Literature cited," p. 953.

its solubility in and absorption from the digestive tract are matters of some little interest in connection with our study of an even more insoluble chlorine compound. Hall (*6*) pointed out in his first paper that the safety of carbon tetrachloride appeared to be correlated with its insolubility. Chopra and McVail (*4*) say: "Owing to its low solubility and volatility and consequently slow rate of diffusion, only small quantities are absorbed into the circulation. Large quantities can therefore be introduced into the alimentary canal without untoward effects." So far as we can judge from the available evidence, the amount of absorption of carbon tetrachloride from the digestive tract does not usually increase as the amount administered is increased, or if it does, there seems to be a compensatory elimination of the larger amounts of the drug without a concomitant and strictly proportionate increase in liver injury. Wells (*11*) has recently shown that a high percentage of the absorbed carbon tetrachloride is rapidly excreted in the expired air.

The experience of various workers indicates that a certain amount of drug is absorbed usually and that it causes a moderate amount of injury to the liver, this injury being rapidly and completely repaired. In fact, Bose and Mukerji (*1*) have reported two cases in which a levulose test of liver function before and after treatment with carbon tetrachloride showed deficiency of liver function before treatment and normal function after treatment, a thing theoretically possible where a chronic indolent pathological condition is rendered acute and stimulated to repair. When large amounts of the drug, 25 c. c. per kilo, or totals of several hundred c. c. at a time, or up to 2 liters in repeated doses, are administered to dogs, as has been done, it seems clear that if the absorption and a concomitant liver injury increased proportionately these animals would be killed in all cases, as we know that animals may be killed with therapeutic doses if conditions are such as to facilitate absorption, for instance, such conditions as appear to be present in certain cases of hemorrhagic enteritis. However, normal dogs commonly survive these heavy doses, showing that absorption or the tissue injury from the absorbed drug is limited; almost all the deaths reported in man and dogs followed therapeutic doses.

Lamson, Gardner, Gustafson, Maire, McLean and Wells (*9*) report that of 35 dogs given 100 to 500 times the therapeutic dose only 1 died. To put it in other words, there is no such thing as a minimum lethal dose of carbon tetrachloride for such animals as man and dog, and the same is true for chickens. Individuals of high individual intolerance for the drug may be killed with therapeutic doses, but normal individuals will tolerate enormous doses, and the limit of tolerance for normal dogs appears to be approximately the limit of gastric capacity. Chloroform, a much more soluble product, soluble 1 part in 161 parts of water at 22° C., is apparently much more readily and regularly absorbed in the digestive tract than is carbon tetrachloride, or a resultant injury is more regularly produced. Caius and Mhaskar (*2*) state in regard to chloroform: "Toxic symptoms are occasionally noticed with a dose of 30 minims [2 c. c.] and become more general and more marked with increasing dosages until 45 minims [3 c. c.] is reached." A dose of 3 c. c. of carbon tetrachloride is the one most used for human adults and usually causes no symptoms or only slight and transient symptoms. Much larger doses have been administered without evident bad effects. Peyre (*10*) has reported no inconvenience from carbon tetrachloride in a case of hepatic cirrhosis with ascites, precisely the sort of case in which bad results would follow if absorption and proportionate injury occurred to any marked extent.

Reasoning from the increased efficacy and safety of carbon tetrachloride as compared with chloroform, and associating it with its lesser water-solubility as perhaps the determining factor for these qualities, the writers have raised the question as to whether a halogen compound less soluble than carbon tetrachloride might be still more effective against hookworms, or safer, or more effective and safer. Carbon trichloride is evidently a less soluble halogen compound. The following experiments were made to test it.

EXPERIMENTAL DATA

COARSE CRYSTALS ADMINISTERED IN CAPSULES

Six dogs were given the coarse crystals in capsules, the number of each dog, its weight, its total dose, and its dose rate in terms of grams per kilo (g. p. k.) being as follows: Dog. No. 665, 13 kilos, 1 gm. (0.077 g. p. k.); No. 666, 10 kilos, 2 gm. (0.2 g. p. k.); No. 667, 6.5 kilos, 3 gm. (0.46 g. p. k.);

No. 668, 9.5 kilos, 4 gm. (0.42 g. p. k.); No. 669, 7 kilos, 1 gm. (0.14 g. p. k.); No. 670, 6 kilos, 6 gm. (1 g. p. k.). These dogs had been fasted overnight previous to the morning of treatment. The feces next day showed no worms, except 3 gravid Dipylidium segments, but all of the feces contained an abundance of the carbon trichloride crystals, the amount seen being such as to suggest that the total amount passed was practically identical with the amount administered. The crystals seemed unchanged in size, shape, and general appearance. The following day there were no worms in the feces except Dipylidium segments, and there were no crystals. Satisfied that carbon trichloride crystals alone were approximately as insoluble in the digestive tract as in water, that they passed through the digestive tract, for the most part, in the course of 24 hours, and that they did not remove worms, the theory that this oil-soluble chemical might be put in contact with worms and its anthelmintic constituent rendered available and effective by the simultaneous administration of castor oil was next tested as follows.

FINE POWDER ADMINISTERED IN CAPSULES WITH CASTOR OIL

Crystals of carbon trichloride were finely powdered in a mortar and administered in capsules, this dose being immediately preceded or followed by 1 ounce of castor oil. The number of each dog, its weight, and its total dose were as follows: Dog No. 680, 7 kilos, 5 gm.; No. 679, 7 kilos, 6 gm.; No. 678, 8 kilos, 7 gm.; No. 683, 10.5 kilos, 8 gm.; No. 682, 9 kilos, 9 gm.; No. 681, 9 kilos, 10 gm. The following day the finely powdered carbon trichloride was visible in the feces. Dog No. 679 passed 1 whipworm the next day and 2 whipworms the day after, a total of 3 whipworms, and No. 680 passed a hookworm the day after treatment. No other worms were passed the fourth day, and on that day the dogs were killed and examined postmortem. The worms present postmortem were as follows: Dog No. 680, 35 hookworms; No. 679, 24 whipworms; No. 678, 5 hookworms, 16 whipworms; No. 683, 25 hookworms, 75 whipworms, 1 Dipylidium; No. 682, 1 hookworm and 1 whipworm; No. 681, negative. Dog 679 passed 3 whipworms and had 24 left; there were 92 whipworms left in the other dogs. Dog 680 passed 1 hookworm and had 35 left; there were 31 hookworms left in other dogs. This experiment suggests that the finely powdered carbon trichloride in the presence of castor oil may become slightly effective against worms, but it indicates that the chemical would probably never be of value as an anthelmintic. To give this feature of oil solubility a more definite test, we tried the carbon trichloride in oil as follows.

CARBON TRICHLORIDE DISSOLVED IN CASTOR OIL

One gram of carbon trichloride was dissolved in 100 c. c. of castor oil with the aid of moderate heat, the solution becoming a brownish amber color. The entire amount was given by drench to dog No. 669. This animal passed no worms the next day, passed 7 whipworms the next day, none the next 2 days, and on post-mortem examination the fourth day after treatment had 14 hookworms, 18 whipworms and 40 to 50 tapeworm heads. The treatment appears to have removed 28 per cent of the whipworms. While little can be concluded from the foregoing experiments as to the effects of oil on the availability of the anthelmintic constituents of the chemical, the results suggest that this highly insoluble drug is entirely ineffective and unavailable when given alone as coarse crystals; that it becomes slightly effective and available when given as a fine powder accompanied by castor oil; and that it becomes still more effective, though by no means really valuable, when it is dissolved in oil and then administered.

Since the efficacy of oil of chenopodium for removing worms from dogs is fairly well known from large numbers of critical experiments, the writers next undertook to test the efficacy of carbon trichloride dissolved in this oil. The oil alone falls distinctly short of 100 per cent efficacy against hookworms in dogs, so that any marked increase in efficacy on the part of the mixture might be detected and would indicate an anthelmintic action on the part of the carbon trichloride.

CARBON TRICHLORIDE DISSOLVED IN OIL OF CHENOPODIUM

Six grams of carbon trichloride were dissolved in 20 c. c. of oil of chenopodium, without heat, and the dose rate for chenopodium for removing hookworms was used for the mixture in treating 6 dogs. This dose rate is 2 c. c. for dogs of average size or larger, and 1 c. c. for small dogs. Two c. c. was given to dogs No. 672 (14 k.) and 673 (12 k.), and 1 c. c. was given to

dogs No. 674 (6 k.), 675 (6.5 k.), 676 (6.5 k.), and 677 (6.5 k.). One ounce of castor oil was given at the same time. The worms passed during the next 4 days, those present post-mortem, and the percentage of efficacy were as follows:

Dog 672 passed 7 hookworms and had 3 post-mortem (70 per cent), passed 27 whipworms and had 1 post-mortem (96 per cent), passed 0 Dipylidium and had 17 post-mortem (0 per cent).

Dog 673 passed 0 hookworms and had 2 post-mortem (0 per cent), passed 2 whipworms and had 26 post-mortem (7 per cent), and passed 0 Dipylidium and had 8 post-mortem (0 per cent).

Dog 674 passed 0 worms and had 1 hookworm, 17 whipworms and 1 Dipylidium sp., an efficacy of 0 per cent for hookworms, whipworms, and Dipylidium.

Dog 675 passed 1 ascarid and had 0 post-mortem (100 per cent), passed 1 hookworm and had 1 post-mortem (50 per cent), and passed 1 whipworm and had 0 post-mortem (100 per cent).

Dog 676 passed 0 worms and had 4 whipworms post-mortem; efficacy 0 per cent for whipworms.

Dog 677 passed 1 hookworm and had 0 post-mortem (100 per cent); passed 0 whipworms and had 101 post-mortem (0 per cent).

The treatment removed 100 per cent of the ascarids present, 56 per cent of the hookworms present, 1.7 per cent of the whipworms present, and 0 per cent of the Dipylidium present. This represents no increase in anthelmintic efficacy over chenopodium alone. Consequently it appears that the solution in oil of an anthelmintic highly insoluble in water does little in the way of rendering the dissolved chemical available as an anthelmintic.

CARBON TRICHLORIDE DISSOLVED IN CARBON TETRACHLORIDE

As a final test carbon trichloride dissolved in carbon tetrachloride was administered at the rate of 25 gm. of the former in 100 c. c. of the latter. The protocols were as follows:

Dog 665: 0.3 c. c. per kilo of solution in capsules; passed 2 hookworms, 10 ascarids, and 16 whipworms; post-mortem, 4 whipworms; efficacy, 100 per cent for hookworms and ascarids, and 80 per cent for whipworms.

Dog 666: 0.3 c. c. per kilo in capsules; passed 1 hookworm and 4 whipworms; post-mortem 5 whipworms; efficacy, 100 per cent for hookworms and 44 per cent for whipworms.

Dog 667: 0.5 c. c. per kilo by capsule; passed 1 whipworm; post-mortem, 0 worms; efficacy, 100 per cent for whipworms.

Dog 668: 1 c. c. per kilo by stomach tube; passed 2 hookworms and 15 whipworms; post-mortem, 0 worms; efficacy, 100 per cent for hookworms and whipworms.

Dog 670: 2 c. c. per kilo by stomach tube; passed 20 hookworms and 4 whipworms; post-mortem, 0 worms; efficacy, 100 per cent for hookworms and whipworms.

The solution of carbon trichloride in carbon tetrachloride was 100 per cent effective against ascarids in the 1 case involved, 100 per cent effective against hookworms in the 4 cases involved, and 100, 100, 80, and 44 per cent effective against whipworms in the 4 cases involved. At the dose rate, which is equivalent to the therapeutic dose rate for carbon tetrachloride (0.3 c. c. per kilo), the solution removed all the ascarids and hookworms and 44 and 80 per cent of the whipworms. This is just about what would be expected from the carbon tetrachloride alone. In the higher rates the efficacy against whipworms rises, as it should theoretically, and as it does frequently in actual practice. Apparently the addition of the carbon trichloride to the carbon tetrachloride does not add to or detract from the anthelmintic efficacy of the latter chemical.

PATHOLOGY

In order to obtain additional information, if possible, in regard to the solubility of carbon trichloride and also its possible toxic effects, portions of the liver and kidneys of dogs Nos. 665, 666, 667, 668, and 670 (receiving carbon-trichloride crystals and afterwards carbon trichloride in carbon tetrachloride), and of No. 669 (receiving carbon-trichloride crystals and afterwards carbon trichloride dissolved in castor oil), were submitted to the Pathological Division, Bureau of Animal Industry, for examination. These tissues were examined by G. T. Creech, who reported that he found in all of them lesions similar to those which have been described by various writers as following the administration of carbon tetrachloride. In the livers there was more or less capillary congestion and the cells showed alterations varying from cloudy swelling to complete degeneration and atrophy of the cells. The kidneys showed hemorrhages, especially in the cortical por-

tions, and the glomeruli and tubular epithelium showed distinct alterations. As the kidneys of dogs commonly show pathologic conditions, little can be concluded from the findings here. That changes along these lines should occur in the case of dog No. 669, which was given carbon trichloride alone, suggests that the chemical had been absorbed to some extent in spite of its great insolubility, but as only one animal was examined in this connection not very much can safely be concluded from this case. It does establish the presumption that some carbon trichloride is absorbed and that it may affect the liver unfavorably.

SUMMARY

Experiments with carbon trichloride administered as coarse crystals in capsules, or as a fine powder in capsules followed or preceded by castor oil, or dissolved in castor oil, or dissolved in oil of chenopodium, or dissolved in carbon tetrachloride, all indicate that carbon trichloride has no value as an anthelmintic. Since the high chlorine content of carbon trichloride would indicate, on theoretical grounds, that this chemical would be quite effective in removing hookworms, the failure of the chemical to display such efficacy in actual practice is correlated with the great actual and relative insolubility of the chemical, the lack of solubility maintaining the anthelmintic chlorine constituent in an unavailable condition.

Considering the solubilities and efficacies of four chlorine compounds of the ethane and methane series, arranged in the order of anthelmintic efficacy, with the most effective first, they are as follows: CCl_4, $CHCl_3$, $C_2H_4Cl_2$, and CCl_3. Their respective water solubilities are: 1 in 1,250; 1 in 161; 1 in 120; 1 in over 10,000. The peak of anthelmintic efficacy is reached at a solubility of 1 : 1,250 (carbon tetrachloride). As the curve of anthelmintic efficacy falls away on the side of increasing solubility, the efficacy decreasing as the solubility increases, it does not appear to promise anything of value in the way of anthelmintic discovery to investigate chlorine compounds of these series which are more soluble than 1 : 1,250, although such investigations have considerable theoretical interest. The anthelmintic efficacy falls to practically zero on the side of decreased solubility for the highly insoluble carbon trichloride. There is still the possibility that a chlorine compound of approximately the same chlorine content as carbon tetrachloride, but slightly less soluble, might be safer if not more effective. Tetrachlorethylene may be such a drug. Evidently carbon trichloride is too far in the direction of insolubility. Judging from these experiments, carbon trichloride is so insoluble that its theoretically effective chlorine content is unavailable and ineffective.

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A STUDY OF THE INFLUENCE OF INOCULATION UPON THE FERMENTATION OF SAUERKRAUT [1]

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INTRODUCTION

The older literature on the subject of sauerkraut fermentation is concerned chiefly with a study of the microorganisms found in kraut without devoting much attention to the kinds and amounts of fermentation products. A review of this literature has been given in a previous publication [2] and need not be repeated here. In papers [3] published from these laboratories the chief fermentation products contained in sauerkraut were found to be: Lactic acid, 0.93 to 1.65 per cent; acetic acid, 0.22 to 0.47 per cent; ethyl alcohol, 0.14 to 0.84 per cent. The variation in composition depends upon many factors such as the age of the kraut, the type of organisms present, and the temperature and completeness of the fermentation.

The success which has attended the use of selected cultures of bacteria in the butter and cheese industry and in the growing of nitrogen-assimilating legumes suggests the use of similar methods in the kraut industry.

Some data were submitted in a previous paper [4] to show that the quality of the kraut and the quantity of the fermentation products could be influenced by the use of selected cultures of lactic acid bacteria. This work has been continued on a larger scale for the past two years and the results obtained are presented in this paper.

EXPERIMENTAL DATA

It was planned to study the chief bacteriological and chemical changes which take place at different times during the fermentation in both an uninoculated and inoculated kraut. The organism selected for the inoculation of the kraut was a strain of *Streptococcus lactis* originally isolated from milk. The use of this culture had consistently given a better quality of kraut than any of the other organisms tried and also better than the uninoculated control.

The shape of the organism, the formation of a smooth firm curd in milk, and its failure to ferment xylose increased the possibility of its detection in kraut. Its chemical products are also distinctive. It has been shown that this strain of *Streptococcus lactis* ferments the common sugars with the formation of active lactic acid and the production of little or no carbon dioxide, alcohol, or acetic acid.

Although some of the results previously reported were obtained on kraut made under factory conditions, much of it was made in large percolators of 3-liter or 4-liter capacity or small 2-gallon stone jars. In order to simulate factory conditions more closely, two large cypress vats about 5 feet high and 2 feet in diameter were constructed. A small well was built on the inside of each vat. This was made by attaching a V-shaped trough, made from two 3-inch boards, to the inside of the vat, so that the brine could rise freely in it. Samples of brine could be taken from these wells at any time during the fermentation period without distrubing the contents of the vat. It was found that as the fermentation proceeded the acidity of the brine in the wells was not the same as that of the brine in the vat, so a hole was bored through the side of the vat about 2 feet from the bottom and samples of the brine were drawn from here through a glass tube inserted through a rubber stopper. In order to obtain the temperature of the brine, a theremometer was suspended deep in the well.

The vats were filled with 250 pounds of cut cabbage at a kraut factory near Madison, Wis. The cabbage used for filling the vats was the same as that used in the factory and was prepared

[1] Received for publication July 26, 1924; issued July, 1925. Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

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in the same manner. While the cabbage was being placed in the vats, 2½ per cent of common salt was added by the regular salter of the factory.

The contents of one vat were inoculated with about 2 liters of a three-day-old culture of lactic acid bacteria in a 1 per cent glucose-yeast-water medium. This culture was diluted with an equal volume of water and applied to the cabbage with a small water sprinkler while the cabbage was being placed in the vat. An equal amount of water was added to the control which received no inoculation.

When filled, the vats were shipped to the laboratory and stored in a room where the temperature was approximately 20° C. Analyses were begun about 24 hours after the cabbage was packed into the vats. Whenever a layer of kraut was removed for an analysis the first 6 inches was discarded before the sample for analysis was taken.

The total number of bacteria present at different stages of fermentation of the kraut were determined by the dilution method, the plate method, and the direct count or Breed method.[5] The organisms present were classified in groups according to their ability to ferment various sugars, mannitol, and litmus milk. Yeast water containing 1 per cent of these compounds was used as a culture medium.

The chemical work consisted of determining the amounts of volatile and nonvolatile acid and alcohol and the total amount of sugar in the brine. The methods used have been described in previous publications.[6]

DETERMINATION OF THE NUMBER OF BACTERIA BY THE PLATE METHOD

To determine the number of bacteria present, glucose-yeast-water agar plates were poured from dilutions of kraut brine made in the usual manner (Table I).

Observations made on the plates at the time of counting showed marked differences in appearance at the various intervals of plating. There was also a noticeable difference in the flora on the plates made from uninoculated kraut. This was most noticeable during the first days of the fermentation. The plates made from the brine 1 day old showed a large number of molds of various types and many different forms of bacteria. Some of these formed large, slimy, raised colonies; some, thin spreading colonies; and others, chromogenic colonies ranging in color from gray or brown to orange and red. The chromogens and molds were present on the plates from both the inoculated and uninoculated kraut but did not appear on plates from dilutions greater than 10,000.

TABLE I.—*Number of bacteria in young sauerkraut as obtained by the plate method*

Age	Number of bacteria in 1 c. c. of brine	
	Uninoculated	Inoculated
Days	*Thousands* [1]	*Thousands* [1]
1	200	170 to 700
3	5,000 to 5,500	3,900 to 4,000
5	6,000 to 6,400	8,000 to 8,200
7	39,000 to 40,000	50,000 to 55,000

[1] Thousands—i. e., 000 omitted.

By the third day the flora had changed and practically only two types of bacterial colonies were present—one a small lens-shaped colony which grew beneath the surface, and the other a surface colony which had a solid center and a hazy border. The uninoculated kraut showed two or three spreading colonies per plate at a dilution of 1,000, but the plates made from the inoculated kraut showed no molds or spreading colonies. The molds, spreading colonies, and chromogens in the natural fermentation persisted for only four or five days. By the fifth day only two types of colonies were present on both control and inoculated plates—the small colony beneath the surface and the large colony on the surface. The small colonies were more abundant than the large colonies, and this ratio was more marked in the inoculated than in the uninoculated kraut.

THE NUMBER OF BACTERIA AS DETERMINED BY DIRECT COUNT OR BREED PLATE METHOD

Each time the vats were opened a sample of brine was removed with a sterile pipette and used for making

[5] AMERICAN PUBLIC HEALTH ASSOCIATION AND ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS' STANDARD METHODS OF MILK ANALYSIS. BACTERIOLOGICAL AND CHEMICAL. Ed. 4, 40 p., illus. New York City. 1923.

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counts by the Breed plate method. The formation of clumps makes it difficult to determine the number of single cells. In many cases clumps of bacteria may have been counted as one cell (Table II).

TABLE II.—*Number of bacteria in sauerkraut as obtained by the direct-count method*

Age	Number of bacteria in 1 c. c. of brine	
	Uninoculated	Inoculated
Days	*Thousands* [1]	*Thousands* [1]
3	55,000	60,000
5	80,000	85,000
7	105,000	145,000
12	115,000	125,000
21	110,000	120,000
30		125,000
73	50,000	50,000

[1] Thousands—i. e., 000 omitted.

The examination of the Breed plates showed, first, a change in the ratio between the types of organisms present in sauerkraut at different times during the fermentation; second, a difference in the flora of the inoculated and uninoculated krauts. During the early part of the fermentation period the inoculated kraut showed about equal numbers of short rods and coccus forms, while the uninoculated kraut contained more rods than coccus types. During the course of the fermentation the number of coccus forms decreased in relation to the number of rod forms. This decrease was most rapid in the uninoculated kraut. The short rods were later replaced by a long rod which was the predominating organism at the end of the fermentation. There was very little difference in the appearance of the flora of the uninoculated and inoculated kraut at the end of the fermentation, because the coccus forms had practically disappeared in both cases.

DETERMINATION OF THE NUMBER AND KIND OF BACTERIA BY MEANS OF SUGARS

Each time when the vats were opened, a small quantity of brine was removed from the vats with a sterile pipette and placed in a sterile flask. The brine was carried through a series of eight dilutions extending from 1 c. c., diluted to 100, to 1 c. c. diluted to 1 billion. From each of these dilutions 1 c. c. was transferred to a tube containing 10 c. c. of sterile yeast water and 0.5 per cent of one of the following compounds: Glucose, lactose, xylose, and mannitol. Besides the sugar media, two sets of tubes containing litmus milk were also inoculated in the same manner. One set of tubes of litmus milk was heated after inoculation to 80° C. for 10 minutes, and then melted vaseline was poured into the tube. All of the tubes were incubated for 96 hours at 28° C. Observations were made for turbidity and gas production after 24, 48, and 96 hours after inoculation. The highest dilution in which there was turbidity or production of gas was taken as the number of organisms present which would ferment that particular substance. After 96 hours the tubes were kept at room temperature, approximately 20° C., for 10 days, and at the end of this time the total acidity was titrated with 0.1 N NaOH.

The litmus milk tubes with vaseline plugs were made to determine if any spore-forming, gas-producing anaerobes were present. Since no gas was produced and the litmus milk was not reduced or curdled, it is believed that none of the above group of organisms was present in the kraut.

Table III has been compiled from more than 300 observations and shows the number of organisms in 1. c. c. of brine that will ferment some of the common sugars, mannitol, and litmus milk. The rate of increase and decrease in the number of organisms is expressed in the same table.

It will be noted that the number of bacteria which ferment any of the substances used increases rapidly during the first 7 days of the fermentation and then decreases. The number of mannitol fermenters is much less than the number of sugar fermenters at the height of the fermentation, but toward the end of the fermentation the numbers are approximately equal. The enormous number of xylose fermenters present in the kraut at the height of the fermentation shows the importance of these bacteria in kraut formation. The influence of inoculation is indicated by the smaller number of xylose fermenters found in the inoculated kraut. This is to be expected because the lactic bacteria used to inoculate the kraut do not ferment xylose.

The figures for litmus milk show that the lactic acid organisms increase much more rapidly in the inoculated than in the uninoculated kraut, and that they are present in much greater

TABLE III.—*Number of bacteria in sauerkraut that ferment various compounds*

[Thousands of bacteria in 1 c. c. of brine [1]]

No.	Medium	Treatment	Age in days						
			1	3	5	7	12	30	73
1	Litmus milk	Uninoculated	1	10	100	100	1	1	0.1
2	do	Inoculated	10	10,000	10,000	100,000	100	100	.1
3	Glucose	Uninoculated	10	10,000	10,000	1,000,000	100,000	1,000	10
4	do	Inoculated	100	10,000	100,000	1,000,000	10,000	10,000	100
5	Lactose	Uninoculated	10	1,000	10,000	1,000,000	10,000	10,000	10
6	do	Inoculated	10	1,000	10,000	1,000,000	100,000	100,000	100
7	Xylose	Uninoculated	10	10,000	10,000	1,000,000	100,000	10,000	100
8	do	Inoculated	10	1,000	10,000	1,000,000	100,000	1,000	10
9	Mannitol	Uninoculated	10	1,000	10,000	10,000	10,000	10,000	100
10	do	Inoculated	10	1,000	10,000	100,000	10,000	10,000	100

[1] Thousands—i. e., 000 omitted.

TABLE IV.—*Reduction and curdling time of litmus milk inoculated with diluted sauerkraut brine*

Kind of kraut	Age of kraut	Highest dilution for reduction and curdling	Reduction time	Curdling time	Kind of kraut	Age of kraut	Highest dilution for reduction and curdling	Reduction time	Curdling time
	Days		*Hours*	*Hours*		*Days*		*Hours*	*Hours*
Uninoculated	1	3	24	48	Inoculated	7	8	48	96
Inoculated	1	4	48	48	Uninoculated	12	3	24	48
Uninoculated	3	4	24	48	Inoculated	12	5	48	96
Inoculated	3	7	48	96	Uninoculated	30	3	48	96
Uninoculated	5	5	48	96	Inoculated	30	5	48	96
Inoculated	5	7	48	96	Uninoculated	73	2	48	48
Uninoculated	7	5	48	96	Inoculated	73	2	48	96

TABLE V.—*Acid production from high and low dilutions of inoculated and uninoculated kraut*

[0.1 N acid in 10 c. c. of culture]

No.	Treatment	Dilution	Age in days							
			1	3	5	7	12	30	73	Average
	Litmus milk:		*C. c.*	*C. c.*	*C. c.*	*C. c.*	*C. c.*	*C. c.*	*C. c.*	*C. c.*
1	Uninoculated	Low	13.3	20.4	20.0	12.8	13.0	12.1	13.8	15.1
2	Inoculated	do	10.9	22.2	18.0	16.2	16.1	12.8	16.8	16.1
3	Uninoculated	High	12.0	15.1	15.5	11.1	13.0	12.1	13.8	13.2
4	Inoculated	do	13.5	10.9	14.4	10.9	12.3	16.6	16.8	13.6
	Xylose:									
5	Uninoculated	Low	5.2	9.0	8.7	9.0	8.5	3.8	7.4	7.4
6	Inoculated	do	5.9	9.8	8.3	8.3	8.5	5.5	9.2	7.9
7	Uninoculated	High	6.7	5.5	6.7	8.4	7.0	4.3	5.1	6.2
8	Inoculated	do	5.0	5.6	6.5	8.9	8.1	6.6	5.1	6.5
	Glucose:									
9	Uninoculated	Low	6.6	7.1	8.6	8.5	6.9	3.4	9.0	7.2
10	Inoculated	do	5.3	7.9	7.1	8.4	7.3	4.5	8.3	7.0
11	Uninoculated	High	4.3	5.2	5.3	5.7	6.1	5.5	8.1	5.7
12	Inoculated	do	4.4	5.0	6.0	6.5	7.5	5.2	7.8	6.1
	Lactose:									
13	Uninoculated	Low	5.5	8.2	7.4	6.8	6.8	5.5	3.0	6.2
14	Inoculated	do	6.7	9.1	6.5	6.1	5.7	5.5	3.7	6.2
15	Uninoculated	High	2.8	4.1	5.3	5.3	6.8	4.7	3.8	4.7
16	Inoculated	do	2.2	4.4	4.7	5.2	6.5	5.0	5.5	4.8

numbers throughout the greater part of the fermentation period. The litmus milk is used as a measure of the number of lactic bacteria which were added, because these organisms form a firm smooth curd, free from gas and without whey. They also reduce the litmus milk before curdling. The highest dilution in which there was reduction followed by curd formation was taken as the number of lactic-acid bacteria present.

It will be seen from the data in Table IV that reduction and curdling of the litmus milk takes place at much higher dilutions with the inoculated brine than with the uninoculated. It will also be noted that in all but two cases the reduction time precedes the curdling time. In these two cases reduction probably preceded curd formation, but observations were not made at short enough intervals to determine exactly when the changes occurred with respect to one another.

ACID PRODUCTION BY THE BACTERIA OF KRAUT

It has been mentioned previously that the solutions of sugars, mannitol, and litmus milk which had been inoculated with diluted kraut brine were titrated with 0.1 N NaOH at the end of 10 days. A great number of titrations were made, but all will not be given here. Table V shows the acidities formed from low and high dilutions of uninoculated and inoculated kraut. This table shows that the acidities developed from any of the substances used is greater when that substance is inoculated with brine from low dilutions than when it is inoculated from high dilution. Individual titrations may show an occasional exception to this rule, but when an average of all the titrations for each substance is taken it will be found to be true. It was also found that for intermediate dilutions the acidities developed were between those for the low and high dilutions in over 80 per cent of the cases.

These data may be interpreted as indicating that in sauerkraut there is a complex flora of organism and that those organisms which are present in largest numbers are not the highest acid producers. Bacteria present in small numbers are eliminated as the dilutions are increased while the more numerous but low acid-producing bacteria remain. Another explanation is the associative action of the bacteria. In the lower dilutions a greater variety of organisms is found than in the higher dilutions. The organisms present in the higher dilutions may be incapable of producing high acidities due to the absence of certain beneficial but non-acid-producing bacteria.

CHEMICAL DATA

The bacteriological data obtained tend to show that the bacterial flora has been influenced by inoculation with selected lactic organisms and that these organisms predominate during the early part of the fermentation. If this is correct, there should be a difference in the composition of the inoculated and uninoculated kraut.

Table VI shows the chief fermentation products found in sauerkraut at different periods during the fermentation. Inoculated kraut shows the presence of the characteristic products of the added lactic-acid culture, especially during the early part of the fermentation. The inoculated kraut is consistently higher in lactic acid and lower in alcohol and acetic acid than the uninoculated kraut. This differ-

TABLE VI.—*Chief products found in sauerkraut at various stages in the fermentation*

Treatment of kraut	Age	0.1 N acid in 100 c. c. of brine	Volatile acid as acetic	Non-volatile acid as lactic	Alcohol as ethyl	Total reducing sugar as glucose
	Days	*C. c.*	*Per cent*	*Per cent*	*Per cent*	*Per cent*
Uninoculated	3	76	0.20	0.58	0.21	1.76
Inoculated	3	74	.17	.60	.17	1.95
Uninoculated	5	104	.30	.76	.24	1.97
Inoculated	5	101	.22	.80	.20	2.08
Uninoculated	7	152	.30	.84	.25	1.90
Inoculated	7	160	.23	.91	.22	1.93
Uninoculated	12	183	.37	1.19	.54	.64
Inoculated	12	174	.36	1.32	.31	.91
Uninoculated	21	193	.31	----------	.62	.34
Inoculated	21	195	.28	1.57	.37	.96
Uninoculated	30	200	.27	1.52	.75	.33
Inoculated	30	197	.34	----------	.53	.72
Uninoculated	73	188	.26	1.50	.60	.23
Inoculated	73	184	.33	1.69	.48	.30

ence is particularly marked in the case of alcohol.

The destruction of sugar is slower in the inoculated than in the uninoculated kraut, although the total acidity is about the same. It is not improbable that fermentation continues for a longer period in the inoculated kraut and thus aids materially in producing a kraut of good flavor as well as high acidity.

Many more determinations could be given to show the influence of inoculation on the fermentation products, but this would be only a repetition of Table VI. This table is representative of the results obtained from more than 50 analyses of inoculated and uninoculated krauts.

QUALITY OF THE KRAUT.—When the vats were opened for the final analysis, the quality of the inoculated and uninoculated kraut, at the age of 73 days, was compared by 10 people who were familiar with sauerkraut. The kraut which had been inoculated was far superior to the uninoculated kraut in regard to flavor and color, although there was not such a noticeable difference in the texture. This observation compares favorably with those made in 50 other cases where the effect of inoculation was studied.

However, the results obtained in this paper must not be construed as implying that the authors recommend the general use of inoculation in the preparation of sauerkraut. The results are suggestive but not final. Many experiments on a factory scale must be conducted to determine not only the possibility but also the practicability of such a procedure.

SUMMARY

Inoculation of sauerkraut with selected cultures of lactic-acid bacteria altered the normal flora of kraut and gave an improved product. It reduced the number of foreign organisms and the duration of their existence in the fermentation.

The presence of the added bacteria was most apparent during the period of most active fermentation and could be detected by direct microscopic examination and by inoculation of litmus milk. A reduction and curdling of the milk, typical of the added bacteria, was obtained.

The effect of inoculation is seen also in the fermentation products. The inoculated kraut contained more lactic acid and less acetic acid, and less ethyl alcohol than the uninoculated kraut.

A PHYSIOLOGICAL STUDY OF MUCOR RACEMOSUS AND DIPLODIA TUBERICOLA—TWO SWEET POTATO STORAGE-ROT FUNGI[1]

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INTRODUCTION

Most of the investigations conducted thus far by the writer and his colleagues on the storage-rot fungi of sweet potatoes have been conducted with the various species of the genus Rhizopus, a group of fungi generally conceded to be very destructive to many vegetables and some fruits (*7*).[2]

Inoculation experiments showed that 27 different hosts were susceptible to decay by some of the species, two of which, *Rhizopus microsporus* and *R. chinensis*, attacked the hosts somewhat weakly. Likewise, the same species, with the exception of *R. microsporus* and *R. chinensis*, were found to cause a soft rot of sweet potatoes (*6*). Numerous isolations from various hosts infected naturally in storage and on the markets gave almost exclusively either *R. nigricans* or *R. tritici*, the former greatly predominating. Subsequent investigations (*11*) demonstrated that these two species were primarily responsible for the decay of sweet potatoes in storage, and at different temperatures in infection chambers, even when brought into competition with other species of the same genus. *Rhizopus nigricans* predominated at temperatures between 6° and 20° C. and *R. tritici* at 30° and above, with some overlapping at temperatures from 20° to 30°.

These organisms produce a watery soft rot and cause the host to decay quickly. Under favorable conditions sweet potatoes of an average size may be decayed in from three to five days. The cells are not entered by the fungus, at least in the earlier stages. Investigations (*4*, *5*) have shown that an enzyme, pectinase, is secreted and has the ability to dissolve the middle lamellae so that coherence of the cells is lost. A portion of this cell wall dissolving enzyme was shown to be exuded into the culture media (*8*), the remainder being retained by the mycelium. When the fungus is grown two or three days on a suitable medium such as sweet potato decoction, raw sweet potato disks 1 mm. thick are completely disintegrated in from two to four hours when immersed in a substrate after freed from the mycelial growth.

The organisms of the Rhizopus group studied both from the standpoint of their parasitism and physiology produce decay at temperatures from about 16° to 30° C. or higher, and cause a rapid destruction of their hosts. They differ essentially in this respect from such fungi as *Mucor racemosus* and *Diplodia tubericola*, the two organisms which form the basis for the discussions in this paper.

Mucor racemosus, although not so common or destructive as *Rhizopus nigricans* or *R. tritici*, is nevertheless frequently found on vegetables, especially sweet potatoes (*3*), when held at temperatures a little above freezing. As a matter of fact, this fungus seems to be of no economic importance except at low temperatures. If, for example, sweet potatoes are held for several weeks at low temperature, from one to several infection centers of *M. racemosus* may develop. This fungus causes a slow progressive rot of sweet potatoes. The tissue is rendered gray and somewhat stringy, but not so wet as by the rot caused by *R. nigricans*. A microscopic examination of the decayed tissue shows that the cells are separated along the line of the middle lamellae in a manner similar to cells of tissue decayed with *R. nigricans* and *R. tritici*.

Diplodia tubericola differs parasitically and physiologically from both *Mucor racemosus* and the various species of Rhizopus. It appears to have a great variety of hosts and to grow

Received for publication July 29, 1924; issued July, 1925.

[1] Reference is made by number (italic) to "Literature cited," p. 968.

saprophytically on almost any woody substance. This fungus is most prevalent in the southern part of the United States, where it is a common cause of a storage rot of sweet potatoes known as the Java black rot. It produces a slow, comparatively dry rot of sweet potatoes, rendering them finally black, hard, and mummified. Investigations have shown that it will cause decay where the humidity of the surrounding air is relatively low. For example, inoculated sweet potatoes will decay if laid on a shelf in a room heated with warm air.

Mucor racemosus and *Diplodia tubericola* seem to differ physiologically and pathologically from the Rhizopus group, with which much of the work thus far has been done. The former causes decay only at low temperatures, and the latter at rather high temperatures and in a relatively dry atmosphere. In view of the striking dissimilarity parasitically between these two organisms and the different species of Rhizopus, they were studied physiologically with the hope of obtaining some facts useful or applicable to the keeping of the sweet potato in storage.

METHODS OF EXPERIMENTATION

The detailed methods employed in conducting the experiments are discussed later, certain modifications and variations being imperative to meet certain phases of the problem, therefore only a cursory discussion of the methods used is given here, certain details being left for discussion when the experimental data are presented.

The culture of *Mucor racemosus* used in these experiments was obtained from sweet potatoes kept at a low temperature at Arlington Farm, near Rosslyn, Va. After this organism had fruited it was "pure-lined" by culturing from a single spore. This pure-lined culture was employed in all the experiments.

The culture of *Diplodia tubericola* was obtained from some decayed sweet potatoes sent to the writer from Alabama in 1922. This organism fruited abundantly in culture and was later proved to be parasitic. It was "pure-lined" in a similar manner to that of *Mucor racemosus*. Both of these organisms were kpet in an active state of vegetative growth by frequent transfers to suitable culture media.

For the enzymatic work several different culture media were employed at various times. The one used for most of the investigations was sweet potato decoction, which medium was prepared according to the following formula: To peeled potatoes add double their weight of water, steam for 1 hour, then squeeze out the liquid through gauze; steam a second time, filter by suction through absorbent cotton and autoclave 20 minutes at 15 pounds pressure. The resulting solution is practically free of cellulose structures, but it does contain some starch and some sugars. These organisms, especially *Mucor racemosus*, make a good growth on this medium.

In studying the secretion and macerating action of pectinase, both the solution on which the fungus grew and the mycelium itself were employed. The fungus was usually grown in 2-liter Erlenmeyer flasks on whatever media were selected for study. The mycelium was carefully removed from the flasks and washed in running water for about 10 minutes, after which the water was squeezed out, the mycelium being subsequently treated with acetone and ether according to a method previously employed by Dox (*2*).

The mycelium after treatment was spread out in order to allow the ether to escape, after which it was bottled and stored until required for use. The mycelium was ground in fine quartz sand before it was employed for macerating experiments. The solution on which the fungus had grown was filtered through absorbent cotton, which removed any mycelium that might be suspended in it, and many of the spores. Toluene was used as an antiseptic. This procedure was frequently unnecessary, inasmuch as maceration was usually complete in a few hours or before any contaminating organisms could alter the results. If the experiment was continued for several hours, toluene was added to the solution. The preparation was then thoroughly shaken and the flask tightly stoppered. Controls prepared by steaming for 10 minutes in an Arnold sterilizer were run with all experiments.

All reducing sugars were determined according to the method of Clark (*1*).

Raw sweet-potato disks were employed in determining the macerating action of the enzyme pectinase. These were cut 1 mm. thick and 15 mm. in diameter from as near the center of a sound sweet potato as possible. Further details of the methods employed may be found in an article by Harter and Weimer (*5*) published in 1921.

EXPERIMENTAL DATA

Enzymes Studied

No attempt has been made to study all or any considerable number of the enzymes which might be produced by

these two fungi. At the outset it was merely planned to study pectinase production, or the secretion and action of the enzyme responsible for the dissolution of the middle lamellae. However, since there was no information available as to any of the enzymes produced by these two fungi, an attempt was made to demonstrate the presence of a few of the more common ones.

PECTINASE

MUCOR RACEMOSUS.—A series of preliminary pectinase experiments with *Mucor racemosus* grown on a modification of Czapek's nutrient solution at room temperature failed to demonstrate the secretion of the enzyme cytase. However, an examination of sweet potatoes decayed by this fungus at temperatures a little above freezing showed that the middle lamellae were dissolved somewhat in advance of the growing hyphae. The fact that decay occurred only at such low temperatures suggested the possibility that either the macerating principle was secreted only at low temperatures, or that it was produced only on certain substrates, or that both hypotheses were true.

Another preliminary experiment in which sweet potato decoction was employed showed that a substance which would completely macerate raw sweet-potato disks in from 6 to 8 hours was secreted into the substrate. A number of different media were later employed as substrates, the results of which will be discussed elsewhere.

A series of experiments was planned subsequently in which after inoculation 2-liter Erlenmeyer flasks containing about 500 c. c. of sweet potato decoction were incubated at a temperature of 5° C. Immediately upon the removal of the cultures from the incubator the substrate and the mycelium were prepared for use in accordance with methods already discussed. As soon as the raw sweet-potato disks were added to the system incubation was carried out at a constant temperature of 35° C.

In the first experiment the organism was grown for 37 days, at the end of which time the flasks were removed from the incubator and the macerating power of the enzyme in the solution on which the fungus grew and that retained by the mycelium was determined. The results showed that maceration of the disks suspended in the substrate was evident in 3 hours, well advanced in 6 hours, and practically complete in 7 hours. The disintegration by the mycelium was very much slower, maceration being only started at the end of 41 hours.

In another series of experiments eight flasks were inoculated at one time and incubated at a constant temperature of 5° C. Three of these flasks were removed from the incubator at the end of 16 days, 3 after 24 days, and 2 at the end of 44 days. The results showed that there was no measurable difference in the time required to completely macerate raw sweet potato disks in the solutions removed from the incubators at the end of the three different periods of growth. Maceration started in about 3 hours and was practically complete at the end of 7 or 8 hours. But the enzyme in 0.25 gm. of mycelium suspended in 25 c. c. of water required 18 to 24 hours to disintegrate the middle lamellae completely. These experiments were repeated several times by growing the mycelium at the same temperature, with no measurable difference in the results.

It has already been pointed out that decay of sweet potatoes by *Mucor racemosus* has been observed to occur only at temperatures a little above freezing. In view of this fact, experiments on pectinase production were first carried out by growing the mycelium at a temperature of 5° C., in which the enzyme was shown to be produced. Some of the enzyme was exuded into the substrate and some was retained by the mycelium.

Inasmuch as decay by this fungus does not usually occur at higher temperatures, experiments were designed to determine whether or not pectinase was produced when the organism was grown at a temperature of 30° C. The methods of experimentation were the same as those already presented for 5°. Without discussing these experiments in detail it may be said that although pectinase was produced at 30°, it was somewhat less active than when the fungus was grown at the lower temperature, for 1 to 2 hours longer were required to macerate raw sweet potato disks completely when immersed in the solution. The action of the enzyme from mycelium suspended in water, however, was considerably slower; in some cases 48 hours or more were required to completely dissolve the middle lamellae so that coherence was lost. It was further found that a considerable amount of the macerating principle was produced during a three days' growth of the fungus, resembling in this respect some of the species of Rhizopus (*9*).

The results of these investigations show that *Mucor racemosus* when grown in sweet potato decoction at 5°

and 30° C. produces a pectinase which, though not as active as that from *Rhizopus tritici*, will macerate raw sweet potato disks.

DIPLODIA TUBERICOLA.—Numerous attempts were made to demonstrate the production of pectinase by *Diplodia tubericola*. This fungus was grown in 10 different substrates, among them sweet potato decoction, and in no case could the secretion of a macerating principle be demonstrated. In this respect the organism differs essentially from the other fungi studied. In view of the negative results obtained, the investigations with this fungus need not be detailed.

AMYLASE

The presence of amylase was demonstrated by the use of potato starch-paste solution to which a weighed quantity of dried mycelium ground in quartz sand was added. Hydrolysis was carried out for 19 hours at a temperature of 35° C. The reducing sugars were determined quantitatively by the method of Clark (*1*). All starch-paste solutions were tested before the addition of the ground mycelium for the presence of reducing sugars, and in no case were any found. A control in which ground mycelium was added to the starch-paste solution and the enzymes inactivated by immediately steaming, was carried in each experiment. The reducing sugars of the control preparations were determined, the amount of sugar found being probably due to autolysis of the mycelium and to such starch digestion as would probably take place before inactivation was accomplished by the steaming. The reducing sugars found were calculated in milligrams per 10 c. c. of solution.

MUCOR RACEMOSUS.—The production of amylase by *Mucor racemosus* was demonstrated by the use of one-fourth gram of the ground mycelium in 50 c. c. of a 0.5 per cent starch-paste solution. The mycelium used was grown on sweet potato decoction for 37 days at a temperature of 5° C. After incubation for 19 hours at 35° the controls averaged 3.14 mgm. and the solutions containing the active enzymes 19.87 mgm. of reducing sugars in each 10 c. c. of solution. This seems to demonstrate clearly that the mycelium contains an enzyme which has the power to hydrolyze starch.

DIPLODIA TUBERICOLA.—The experiments with *Diplodia tubericola* were conducted the same as with *Mucor racemosus*, except that a 2 per cent starch-paste solution was used with 0.5 gm. of powdered mycelium in 50 c. c. of solution. The mycelium was grown for 8 days on Czapek's modified solution at 30° C. The controls averaged 2.133 mgm. and the active enzyme preparation 52.96 mgm. of reducing sugars for each 10 c. c. of solution.

INVERTASE

The production of invertase by *Mucor racemosus* and *Diplodia tubericola* was determined by the use of mycelium from the same source as that employed in the demonstration of amylase. The amount of saccharose hydrolyzed was measured quantitatively by a determination of the reducing sugars.

MUCOR RACEMOSUS.—To 50 c. c. of a 0.5 per cent saccharose solution was added one-fourth gram of powdered mycelium. After incubation for 19 hours at 35° C. the controls averaged 1.42 mgm. and the active enzyme preparations 16.47 mgm. reducing sugars per 10 c. c. of solution.

DIPLODIA TUBERICOLA.—With this organism 0.5 gm. mycelium in 50 c. c. of a 2 per cent saccharose solution was used. At the end of the incubation period the controls averaged 6.09 mgm. and the active enzyme solution 175.88 mgm. reducing sugars per 10 c. c. of solution. The amount of reducing sugars in the control is somewhat larger than that usually found, which may possibly be accounted for by autolysis, or by some of the saccharose having been broken down in heating to inactivate the enzyme, or both of these reasons may be adduced. The sugar solution alone before the powdered mycelium was added or heated contained only 0.95 mgm. of reducing sugars.

RAFFINASE

The secretion of raffinase by *Mucor racemosus* and *Diplodia tubericola* was demonstrated by the hydrolysis of raffinose by the powdered mycelium. One-fourth of a gram of the powdered mycelium was suspended in 50 c. c. of 0.5 per cent raffinose solution and incubated for 21 hours at 35° C. A steamed control was carried at the same time. At the end of the incubation period further hydrolysis was stopped by steaming the solutions in an Arnold sterilizer. The reducing sugars were determined quantitatively.

MUCOR RACEMOSUS.—The controls gave an average of 3.55 mgm. and the active solutions 13.75 mgm. of reducing sugars per 10 c. c. of solution, thus demonstrating the presence of raffinase.

DIPLODIA TUBERICOLA.—The enzyme raffinase, secreted by this organism, was able to hydrolyze more than twice as much of the sugar as that of *Mucor racemosus* in the same length of time. Only 0.71 mgm. of reducing sugars was obtained from the control, while an average of 31.28 mgm. was obtained per 10 c. c. of solution in the preparation containing the active enzyme.

CYTASE

DIPLODIA TUBERICOLA.—It was previously pointed out that *Diplodia tubericola* caused a slow dry-rot of the sweet potato, the tissue finally becoming hard and mummified. The cells of the decayed tissue do not separate in the same way as when the rot is caused by *Mucor racemosus* or *Rhizopus nigricans*. It was also shown that the enzyme pectinase, which is abundantly produced by Mucor and Rhizopus, is not produced by *D. tubericola*, at least within the limits of these experiments. These results, together with the fact that hyphae have been observed within the cells, suggested the possibility that the enzyme cytase may be produced.

The first attempt to demonstrate the production of cytase was made with mycelium grown on sweet potato decoction. The mycelium was treated in the usual way and added to a suspension of cellulose in water. The cellulose was prepared from filter paper according to the method of Scales (*12*). One gram of powdered mycelium was added to 50 c. c. of the cellulose suspension and incubated at 35° C. At the end of 48 hours the system was tested for reducing sugars and none were found. Another method used successfully by Kellerman (*10*) to demonstrate the production of cytase by *Penicillium pinophilum* was tried. A beef agar with cellulose added was used as a medium. This preparation in test tubes was inoculated with *Diplodia tubericola* on the surface and incubated at a constant temperature of 30°. These cultures were grown for several weeks, but there was no clearing of the agar. On the other hand, *Sclerotium rolfsii*, used as a control, cleared the agar almost to the bottom of the tube in the same length of time. The experiment was repeated, using carrot and sweet potato agar with cellulose added. After inoculation the cultures were held for a number of weeks at 30°, but there was no evidence of the production of cytase. In this experiment water was placed in the incubator in order to prevent the media from drying out too rapidly.

MUCOR RACEMOSUS.—This fungus produced no cytase when tested according to the above method.

INFLUENCE OF SUBSTRATE ON PECTINASE PRODUCTION, DRY WEIGHT, AND HYDROGEN-ION CONCENTRATION

Essentially the same methods were employed for both *Mucor racemosus* and *Diplodia tubericola*, some variations being necessary to meet the different habits of the two fungi. *M. racemosus* was incubated at 25° C. and *D. tubericola*, being a higher temperature form was incubated at 30°. Since the latter organism develops much more slowly, it was grown for a longer time.

The following culture media were employed: Prune, sweet potato, potato, carrot, turnip, and bean decoctions, beef bouillon, and Czapek's, Pfeffer's, and Richard's solutions. Czapek's solution was modified by the substitution of ammonium nitrate for sodium nitrate. Enough of each of the stock solutions was prepared at one time to carry out the duplicated experiments. The experiments were set up as follows: Fifteen 100 c. c. flasks were used for each organism, 50 and 35 c. c. of the medium being used in each flask for *Mucor racemosus* and *Diplodia tubericola*, respectively. As soon as the culture media were pipetted into the flasks they were autoclaved for 20 minutes at 15 pounds pressure. They were then held for a few days in the laboratory at room temperature. As soon as it was evident that none of the flasks were contaminated, 10 of each medium were inoculated and incubated at temperatures given above, the remaining 5 being covered with oiled paper to prevent evaporation and held as controls, that is, for hydrogen-ion determinations.

MUCOR RACEMOSUS.—At the end of five days' growth three flasks of each medium were removed and the contents of each made into one compound sample. Two flasks were then prepared from this solution, one of which, after steaming 10 minutes to inactivate the enzyme, served as a control. Raw sweet potato disks were added to the solution and a record made from time to time of the progress of maceration. None of the disks in the steamed controls were macerated. The production of pectinase was not entirely what might be expected. It was abundantly secreted on sweet-potato and string-bean decoctions after 23 hours, on carrot decoctions after 30 hours; and well advanced on turnip decoction after 30

hours. None was secreted on prune or potato decoction or when the fungus was grown on beef bouillon or on Czapek's, Pfeffer's, or Richard's solution. These latter media have consistently failed to stimulate the production of the enzyme. This result is not so surprising, since the synthetic media are not supposed to contain any of the pectic compounds, which have been found to exercise a more or less regulatory influence on the secretion of pectinase.

Eight days after inoculation the remaining seven flasks were taken off. The mycelium from the flasks of the same substrate was brought together and used for dry-weight determinations. The total dry weight of the mycelium from these seven flasks proved to be as follows (average two experiments): Prune decoction, 0.2632 gm., sweet-potato decoction, 0.5668 gm.; carrot decoction, 0.3224 gm.; potato decoction, 0.3513 gm.; turnip decoction, 0.4845 gm.; string-bean decoction, 0.3684 gm.; beef bouillon, 0.2502 gm.; Czapek's solution, 0.4253 gm.; Pfeffer's solution, 0.3600 gm.; Richard's solution, 0.4476 gm. These data show that a fair growth of mycelium was produced in all the culture media used. However, there seems to be very little if any correlation between the amount of dry material and the production of pectinase. The smallest amount of dry material was produced on prune decoction and in beef bouillon, the media in which pectinase was not secreted. On the other hand, Richard's and Czapek's solutions, in which pectinase is not secreted, produced a considerable quantity of dry matter.

The hydrogen-ion determinations were made of the used and unused solutions at the end of the growth period on the same day the solutions were removed from the incubator. The control flasks, which were wrapped over the top with oiled paper to prevent evaporation, were kept in the incubator beside the inoculated ones. When required for hydrogen-ion determinations the contents of the different flasks of each medium were collected into one compound sample. The hydrogen-ion concentration of all the vegetable decoctions, except prune, also that of beef bouillon, is decreased by an 8-day growth of *Mucor racemosus*, but that of Czapek's, Pfeffer's, and Richard's solutions is increased or left practically unchanged (Table I).

DIPLODIA TUBERICOLA.—The same media were used in studying this organism as were used with *Mucor racemosus*. No pectinase was produced by *Diplodia tubericola* on any of the media used, and in view of that fact none of these data will be given.

TABLE I.—*Hydrogen-ion concentration of the controls and used solutions after 8 days' growth of Mucor racemosus. Average of two experiments*

Solution	Control (P_H)	Inoculated solution (P_H)
Prune decoction	3.795	3.645
Sweet-potato decoction	5.075	7.28
Carrot decoction	4.965	7.42
Potato decoction	5.525	7.99
Turnip decoction	4.865	7.455
String-bean decoction	4.625	7.58
Beef bouillon	7.18	8.445
Czapek's solution	5.08	3.485
Pfeffer's solution	3.71	3.76
Richard's solution	3.595	3.60

For the determination of the influence of the medium on dry-weight production and hydrogen-ion concentration the cultures were incubated for 10 days at 30° C. The same methods of procedure for the determination of dry weight of the mycelium and of the hydrogen-ion concentration were followed as with *Mucor racemosus*. The results of these total dry-weight tests (average of two experiments) are as follows: Prune decoction, 3.925 gm.; sweet-potato decoction, 0.7200 gm.; carrot decoction, 1.4498 gm.; potato decoction, 0.4232 gm.; turnip decoction, 1.9966 gm.; string-bean decoction, 0.8683 gm.; beef bouillon, 0.9886 gm.; Czapek's solution, 1.9882 gm.; Pfeffer's solution, 2.4475 gm.; Richard's solution, 1.0316 gm.

The amount of dry matter produced by *Diplodia tubericola* varied in the different media. The largest amount of dry material was produced in prune decoction, in which *Mucor racemosus* made a rather small growth. These data show that a medium which is well suited for the growth of one organism may not necessarily be suited for the growth of another.

To ascertain the hydrogen-ion concentration of the solutions, the contents of all the flasks of the same medium were collected into one compound sample and the P_H value determined as for *Mucor racemosus*. The results are given in Table II.

From Table II it is seen that *Diplodia tubericola* decreased the hydrogen-ion concentration of all the vegetable decoctions and of beef bouillon. The hydrogen-ion concentration of the three synthetic media (Pfeffer's, Richard's, and Czapek's) was increased.

TABLE II.—*Hydrogen-ion concentration of the controls and the used solutions after 10 days' growth of Diplodia tubericola. Average of two experiments*

Solution	Control (P_H)	Inoculated solution (P_H)
Prune decoction	3.86	3.98
Sweet-potato decoction	4.66	8.49
Carrot decoction	5.11	8.62
Potato decoction	6.04	8.72
Turnip decoction	4.98	8.47
String-bean decoction	4.75	8.62
Beef bouillon	7.15	8.59
Czapek's solution	3.77	2.52
Pfeffer's solution	3.62	2.42
Richard's solution	3.65	2.11

DISCUSSION

The foregoing data show that within the limits of these experiments *Diplodia tubericola* does not produce a macerating principle at any of the temperatures tried. On the other hand, *Mucor racemosus* secretes an enzyme which will completely disintegrate the tissue of raw sweet potato disks in from 6 to 8 hours. The same media were used in these experiments as were employed in studying the influence of the substrate on pectinase production by *Rhizopus tritici* (*8*). In the case of the latter organism dissolution of the middle lamellae was complete in a shorter period of time. However, a comparison of the results of the investigations of these two organisms with respect to the time elements is not strictly justifiable, in view of the known differences in the growth habits of the two fungi. It is interesting to note, however, that both fungi are alike influenced by the substrate on which they grow.

Pectinase was produced in all of the vegetable media tried except prune and potato decoction. None was produced by either *R. tritici* or *M. racemosus* when grown on synthetic media (Czapek's, Pfeffer's, and Richard's), or on beef bouillon where glucose was used as a source of carbon.

The only conclusion seemingly to be drawn from these results is that there is a regulatory influence of the substrate. Were it not for the results obtained from prune and potato decoctions it might be assumed on theoretical grounds that the regulatory substance may be some of the pectic compounds. In the article cited above it was shown that although no pectinase was produced by *R. tritici* when grown in Czapek's nutrient solution with glucose as a source of carbon, it was secreted if pectin was supplied as the only source of energy. On the other hand, if the pectin were combined with glucose as the available supply of carbon, the action on raw sweet potato disks was very feeble.

The volume of growth can hardly be urged as a possible explanation, inasmuch as it has been found that when the macerating principle is secreted, this occurs very early in the growth of the fungus, before any considerable amount of mycelium has been produced. A demonstrable amount of pectinase is secreted by *R. tritici* in 6 to 7 hours of growth and by *Mucor racemosus* in 3 days. Although the growth on prune decoction was less than on the other vegetable media employed, it was nevertheless fairly good, and on potato decoction it was not exceeded to any considerable extent except on sweet potato and turnip. The dry weight of mycelium produced on the synthetic media was equal on an average to that on the vegetable media, and no pectinase was produced.

A comparison of Tables I and II shows that both *Mucor racemosus* and *Diplodia tubericola* have somewhat similar action on the hydrogen-ion concentration of the substrate. Prune decoction was not materially changed, but the acidity of all the other vegetable decoctions and of beef bouillon was decreased, and in all cases to the alkaline side of neutrality. The hydrogen-ion concentration of Czapek's, Pfeffer's, and Richard's solutions was increased by *D. tubericola*. *M. racemosus* caused no appreciable change in the hydrogen-ion concentration of Pfeffer's and Richard's solutions, but increased the acidity of Czapek's solution. These results agree only in part with those obtained with *Botrytis cinerea* (*13*), which increased the hydrogen-ion concentration of some of these vegetable media and decreased that of others.

A study of these data and those of *Rhizopus tritici* show that different fungi act differently under what may be considered similar conditions. One organism may increase and another decrease the hydrogen-ion concentration of the same medium. From these results it is evident that no sweeping generalizations can be made for all fungi from the results obtained from a few. Each organism must be assumed *a priori* to be physiologically different from all others.

The investigations already carried out and discussed above show that pectinase was produced when *Mucor*

racemosus was grown on sweet potato, carrot, and turnip decoctions, but not when grown on potato decoction. Only sweet potato tissue was used for macerating experiments, and it was found that it was not macerated when suspended in a solution of potato decoction in which *Mucor racemosus* had grown. It was suspected that the enzyme might be specific in that the potato would be decayed if the organism was first grown on potato decoction.

Sound sweet potatoes, potatoes, carrots, and turnips were inoculated by the well method from a four-days' old growth of *Mucor racemosus* on sweet potato and potato decoction. After inoculation the hosts were kept at a constant temperature of 5° C. for 32 days, then removed from the incubator and examined. All of the sweet potatoes were partially decayed and isolations gave pure cultures of *M. racemosus*. None of the carrots, turnips, or potatoes were decayed, which shows that *M. racemosus* is not parasitic on them, at least within the limits of these experiments. Whether or not potato decoction was used made no difference.

It has already been shown that *Mucor racemosus* when grown on turnip and carrot decoction secretes into the solution a substance which will macerate raw sweet potato tissue, although the fungus is not parasitic on either carrot or turnip. In view of this fact an experiment was outlined for the purpose of determining if when grown on sweet potato decoction a substance is produced which will macerate raw turnip and carrot disks. These experiments were carried out in the usual way by the use of 2-liter Erlenmeyer flasks, which after inoculation were incubated at 10° C. After 14 days of growth the solutions were freed of mycelium by filtering through absorbent cotton. Disks of raw sweet potato, carrot, and turnip 1 mm. thick were suspended in the solutions and incubated at 35°. The results showed that maceration was started in every case in about 3½ hours and was nearly complete in 5 hours. It is evident from these investigations that *M. racemosus* will secrete into the substrate a substance which will dissolve the middle lamellae of hosts of which it is not a parasite.

SUMMARY

The secretion of pectinase by *Mucor racemosus* which causes a rot of sweet potatoes at a temperature of 5° C. and lower, and by *Diplodia tubericola*, the cause of a slow dry-rot at higher temperatures, was tried on 10 different culture media. *D. tubericola* did not secrete the enzyme. *M. racemosus* produced it on certain vegetable decoctions (sweet potato, carrot, turnip, string bean), but not on prune or potato decoction, or on synthetic media (Czapek's, Richard's, Pfeffer's) and beef bouillon.

Both *Mucor racemosus* and *Diplodia tubericola* secreted the enzymes, amylase, invertase, and raffinase, but not cytase.

The influence of these two fungi on the hydrogen-ion concentration of the 10 substrates used was investigated with the following results: *M. racemosus* decreased the hydrogen-ion concentration of sweet-potato, carrot, potato, turnip, and string-bean decoction, and of beef bouillon, and increased that of prune decoction and of Czapek's modified nutrient solution. There was no appreciable change of Pfeffer's and Richard's solutions. *D. tubericola* decreased the hydrogen-ion concentration of all of the vegetable decoctions (prune, slightly) and of beef bouillon. The hydrogen-ion concentration was increased when grown on Czapek's, Pfeffer's, and Richard's solutions.

Mucor racemosus was found to be parasitic on sweet potatoes, but not on turnips, carrots, or potatoes. The growth of this organism on potatoes did not produce an enzyme which would macerate potato tissue. Although not parasitic on turnips and carrots, an enzyme which would macerate sweet-potato disks was produced when grown on decoctions made from these vegetables. When the organism was grown on sweet-potato decoction an enzyme which would macerate turnip and carrot tissue was secreted.

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MICROORGANISMS IN DECOMPOSING OYSTERS [1]

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INTRODUCTION

In a previous paper on the spoilage of oysters (*6*)[3] the total aerobic counts of bacteria were given for shucked oysters in various stages of decomposition. The results of that work indicated that there was no definite correlation between the total number of bacteria present and the stage of decomposition of the oysters. It was shown, however, that there was a definite correlation between the hydrogen-ion concentration of the oyster liquor and the stage of spoilage. Realizing that the spoilage of oysters was probably due to the presence and development of bacteria of certain groups and that after identification of the groups responsible for the spoilage it might be possible to establish a correlation between a differential count of bacteria of certain types and the stage of decomposition, it was apparent that the next step in the investigation was to collect and study, for identification, the bacteria present in decomposing oysters. This paper gives the results of a study of several hundred cultures collected from the dextrose-agar shake cultures and from the dextrose-agar and wort-agar plates made in the course of the investigation already reported (*6*).

NOMENCLATURE

The nomenclature for bacteria, as presented in Bergey's Manual of Determinative Bacteriology (*11*) by a committee of the Society of American Bacteriologists, is used throughout this report. An attempt has been made to identify the organisms to species, but, owing to the meager and inadequate descriptions often found in the literature and to the reactions of the peculiar atypical forms frequently isolated from material of the kind studied here, it has not been possible in all cases to definitely identify the organisms by specific names. The names given to some of the less common forms encountered, therefore, are those of the types most nearly resembled.

The studies of Levine (*8*), of Winslow, Kligler, and Rothberg (*15*), and of many others on the lactose-fermenting group of bacteria, the work of Edson and Carpenter (*2*) and of Tanner (*12*) on the fluorescent bacteria, the investigations of Wenner and Rettger (*14*) and of Bengtson (*1*) on the proteus group, and of Ford and his coworkers (*7*) on the aerobic spore-bearing bacteria, make it possible to identify rather definitely members of these groups. The common water and soil forms belonging to the genera Achromobacter, Serratia, and Flavobacterium do not lend themselves so readily to identification, and it is in these genera particularly that no claim is made that the organisms so named are actually true to type.

EXPERIMENTAL WORK

In collecting the cultures from the dextrose-agar and wort-agar plates, many duplicates were inevitably obtained. In order to eliminate these duplicates, the organisms were studied as to their morphology, Gram staining reaction, growth on agar and gelatin, and reactions in litmus milk and in dextrose, lactose, maltose, and sucrose broths. After the elimination of the duplicates, for the purpose of more definite identification, the following characters of the cultures were also determined: Motility; indole production; reduction of nitrates; fermentation of additional carbohydrates, whenever necessary; reaction to the methyl-red test and the Voges-Proskauer test, whenever significant; growth on potato; and the production of hydrogen sulphide. An accurate record was kept of the history of each organism in order that some data might be available on the source and treatment of the oysters from which the organism was isolated, its relative abundance in the decomposing oysters, and the stage of spoilage at which it was isolated.

[1] Received for publication August 1, 1924; issued July, 1925.
[2] Thanks for valuable suggestions and criticisms are expressed to Charles Thom, Mycologist in Charge, Microbiological Laboratory.
[3] Reference is made by number (italic) to "Literature cited," p. 975.

Several experiments were conducted to investigate the power of the various pure cultures to produce typical spoilage in raw shucked oysters.

Clean oysters in the shell, obtained from unpolluted sources, were "floated" in the laboratory in tanks of artificial sea water to which had been added calcium hypochlorite in sufficient quantity to produce 6 parts of free chlorine per 1,000,000 parts of water. Following the directions given by Wells (*13*) for artificial purification of oysters, an attempt was made on a laboratory scale to reduce the bacterial content of the oysters to a minimum. After a suitable period in the chlorinated water, the oysters were opened aseptically, and one or two oysters, with their liquor, were placed in each of a sufficient number of large sterile test tubes. These chlorinated oysters were then inoculated with saline suspensions of the various pure cultures in the collection, grown for 48 hours on dextrose-agar slants. The inoculated oysters were held at 20° to 25° C., and daily records were made of their odor, appearance, and hydrogen-ion concentration. This experiment was repeated several times. Although not entirely successful, it furnished data which, when considered with some supplemental results from another experiment, presumptively identified those organisms in the collection which were responsible for the spoilage of shucked oysters.

In order to confirm the results obtained in these inoculation experiments, an oyster-infusion broth was prepared from chopped oysters, tubed, and sterilized in the autoclave. Before sterilization a small piece of oyster meat was added to each tube of plain oyster infusion. These tubes were inoculated with the various pure cultures, and the same series of observations made in the inoculation experiments were recorded.

EXPERIMENTAL RESULTS

From the combined results of these several experiments, it was apparent that certain of the bacteria used produced foul, putrefactive odors in the oysters, some produced acidity or sour odors, and others apparently produced neither putrefaction nor souring in pure culture, although they grew readily.

Table I gives the types of bacteria isolated from decomposing oysters which, when inoculated into artificially purified oysters or into oyster infusion medium, produced foul and putrefactive odors. Table II shows the types of organisms from oysters which produced acidity or sourness in oysters and in oyster infusion medium. Table III includes the remaining organisms isolated from decomposing oysters which apparently had no effect in pure culture.

The data in Table I show that the bacteria producing foul odors in decomposing oysters are members of the genera [4] Serratia (water and soil bacteria producing red pigment), Pseudomonas (soil and water bacteria producing a blue-green pigment), Proteus, Clostridium (spore-forming obligate anaerobes), and Bacillus (aerobic spore-forming bacteria). The first four groups were always present during the course of the spoilage and were always comparatively abundant and active. The spore-forming aerobic bacteria were always obtained during the early stages of the decomposition, and they did not appear to be as abundant or as active in producing evidences of spoilage as were the other organisms named in Table I.

The organism designated in Table I as CS–8c, which evidently belongs to the genus Serratia, has been isolated repeatedly, not only in this investigation but in an investigation on the decomposition of salmon (*5*). Since this organism appears to be a particularly active agent in the decomposition of fish and shellfish and since it can not be identified as any member of the genus Serratia described in the literature available, its morphology and cultural reactions may properly be given here. This organism is a Gram negative motile rod with bipolar flagella, 1.8 to 3 microns long and about 1 micron wide. Gelatin colonies are small, white, and perfectly round. Agar colonies are round, of medium size, and yellowish in the center, with bluish edges. Gelatin is rapidly liquefied, the growth being mostly on the surface of the stab. On agar slants the organism grows along the streak; it is pinkish, moist, and slightly raised. There is turbidity in plain broth, with a very slight scum and very little sediment in 48 hours. In old broth cultures there is a heavy viscous sediment, with a definite ring around the tube at the surface of the broth. Litmus milk is reduced and slowly peptonized, with the evolution of a foul odor. On potato the growth is heavy, moist, glistening, brick red, and slightly raised.

[4] Nomenclature follows Bergey's Manual (*11*).

TABLE I.—*Organisms producing foul odors in oysters and oyster infusion medium*

Culture (CS)	Number of times isolated[a]	Stage of spoilage when isolated	Species resembled
8c	29	Present throughout whole period of spoilage.	Serratia sp. (Bizio).
4G	17	do	Pseudomonas fluorescens (Flügge).
2C	27	do	Proteus vulgaris (Hauser).
1P	6	do	Bacillus cereus (Frankland).
3AN	20	do	Clostridium sp. (Prazmowski).
$5A_1$	24	do	Do.
$5A_4$	15	do	Do.
$6A_1$	8	do	Do.
1F	8	Present during early stages of spoilage	Bacillus subtilis (Ehrenberg) Cohn.
9G	2	do	Bacillus aterrimus (Lehmann and Neumann).
7	11	do	Bacillus simplex (Gottheil).
12A	2	do	Bacillus prausnitzii (Trevisan).

[a] In picking colonies from plates an effort was made to pick all the representative colonies from every plate examined.

There is no fermentation of dextrose, lactose, sucrose, or maltose. Indole is not produced. Nitrates are reduced to nitrites. Hydrogen sulphide is produced.

Four spore-forming obligate anaerobes were isolated in the course of the investigation. These organisms resembled one another closely, but were carried through the investigation as individual organisms. It has not been possible to identify these anaerobic bacteria as to species, but it has been determined that they are active agents in producing spoilage in oysters.

Although decomposing shucked oysters have a foul and putrefactive odor, their predominating odor and their appearance are such that they are usually referred to as "sour" oysters. In Table II are listed the organisms which produce this sour odor or produce acidity as indicated by the hydrogen-ion concentration.

All the organisms in Table II, in pure-culture inoculation of oysters and oyster infusion medium, produced hydrogen-ion concentrations varying from P_H 5.6 to P_H 4.4. *Aerobacter aerogenes* (*Bact. aerogenes*) and *A. cloacae* (*Bact. cloacae*) were present throughout the whole period of the spoilage. In pure culture these organisms produced not only a sour odor and an increase in acidity but also a foul and somewhat putrefactive odor. *Escherichia coli* (*Bact. coli*) and *E. communior* (*Bact. communior*) were isolated only during the early stages of spoilage. In pure-culture inoculation experiments these organisms produced results very similar to those produced by *A. aerogenes* and *A. cloacae*.

During the late stages of spoilage the organisms predominating in the spoiled oysters are streptococci, lactobacilli, and yeasts. In pure-culture inoculations the streptococci produce

TABLE II.—*Organisms producing acidity, sour odor, or both, in oysters and oyster infusion medium*

Culture (CS)	Number of times isolated	Stage of spoilage when isolated	Type resembled
7L	71	Present throughout whole period of spoilage	Aerobacter aerogenes (Escherich).
2N	21	do	Aerobacter cloacae (Jordan).
10E	21	Present during early stages of spoilage	Escherichia coli (Escherich).
14E	13	do	Escherichia communior (Durham).
20E	16	Present during late stages of spoilage	Lactobacillus sp. (Beijerinck).
$7A_{20}$	2	do	Streptococcus sp. (Rosenbach).
10Q	25	do	Streptococcus lactis (Lister).
$13A_3$	1	Present during early stages of spoilage	Bacillus mycoides (Flügge).
20c	5	Abundant during late stages of spoilage	Yeasts.
16P	5	do	Do.
16S	4	do	Do.
17A	4	do	Do.
1Z	4	do	Do.
NY_4	1	Isolated during late stages of spoilage	Do.
$20A_4$	2	do	Yeastlike fungus.

no pronounced odors, but they bring about a marked increase in acidity. The lactobacilli produce both a sour odor and an increase in acidity. These organisms, isolated from sour oysters, have been found to ferment dextrose, lactose, sucrose, maltose, arabinose, and trehalose. Some yeasts and yeastlike forms produce no odor in oysters and oyster infusion medium in pure culture. Some of the yeasts isolated do not ferment any of the carbohydrates and apparently play no part in the spoilage of oysters. The yeasts included in Table II, however, produce sour, yeasty, or aromatic odors, as well as an increase in acidity in the oysters. Owing to the difficulties in such work, no attempt has been made to identify the yeasts beyond an examination for ascospore formation. From the observations made it is apparent that most of the yeasts belong to the group of Torulae.

spoilage. Others, which were present at the beginning of the experiment, either disappeared or were overgrown by other groups. The yeasts were usually obtained during the late stages of spoilage. Although the organisms in this table apparently have no effect on oysters in pure culture, it is not improbable that under natural conditions they supplement the activities of the other organisms present in bringing about decomposition.

In studying oysters from polluted and unpolluted sources, Fuller (*4*) found that the bacterial flora of the oysters was almost identical with that of the sea water from which they were taken. His work and the earlier works of Wood (*16, p. 759–764*), Foote (*3*), and Sabatier, Ducamp, and Petit (*10*) are in close agreement with the findings of the investigation reported here. The identity of the flora of the oysters and of the sea water is often destroyed

TABLE III.—*Organisms producing only slight "off" odors or no objectionable odors in oysters or oyster infusion medium*

Culture (CS)	Number of times isolated	Stage of spoilage when isolated	Type resembled
2M	45	Present throughout whole period of spoilage.	Achromobacter raveneli (Chester).
1H	2	do	Achromobacter superficialis (Jordan).
3M	26	do	Achromobacter inunctum (Pohl).
6E	2	Isolated during early stages of spoilage	Achromobacter tiogense (Wright).
15L	1	Isolated during late stage of spoilage	Achromobacter stoloniferum (Pohl).
17c	7	Present throughout whole period of spoilage.	Achromobacter solitarium (Ravenel).
$1A_{13}$	1	Isolated during late stage of spoilage	Eberthella sp. (Castellani and Chambers).
2K	12	Present throughout whole period of spoilage.	Eberthella leporis (Sternberg)
3R	2	Isolated during late stage of spoilage	Flavobacterium aurantinum (Hammer).
3A	2	Isolated during early stage of spoilage	Flavobacterium sp.
7C	1	do	Flavobacterium annulatum (Wright).
9A	3	do	Flavobacterium sulfureum (Zettnow).
$14A_4$	1	do	Flavobacterium rigensis (Bazarewski).
17P	1	do	Flavobacterium deciduosum (Wright).
3B	14	Present throughout whole period of spoilage.	Micrococcus candicans (Flügge).
$5A_9$	1	Isolated during late stage of spoilage	Staphylococcus citreus (Passet).
$10A_{12}$	2	Isolated during early stage of spoilage	Micrococcus subflavescens.
$17A_1$	1	do	Micrococcus freudenreichii (Guillebeau).
1U	2	Isolated during late stages of spoilage	Pink yeast.
IX	1	do	Do.
19F	1	do	Do.
$19A_3$	1	Isolated during late stage of spoilage	Yeasts.
21D	1	do	Do.
NY_5	5	do	Do.
19E	1	do	Yeastlike fungus.
$13A_2$	1	Isolated during early stage of spoilage	Actinomyces.

The organisms in Table III, which were isolated from oysters and yet have no pronounced effect when inoculated into oysters or oyster infusion medium, are for the most part ordinary nonspore-forming water and soil bacteria. Some of these organisms persisted throughout the whole period of

by handling under unsanitary conditions.[5] The organisms which have been found in oysters, some of which bring about spoilage, are common water and soil organisms. This accords with the conclusions reached regarding the source and characteristics of the bacteria causing decomposition in salmon.[5]

[5] Personal communication from P. B. Parsons, Bureau of Chemistry.

It is well known that oysters contain carbohydrate in the form of glycogen. Very few of the organisms isolated and studied in this investigation were able to attack pure glycogen. A great many of them, however, were able to ferment dextrose, maltose, or both. Mitchell (*9*) stated that, with approaching death, glycogenolysis takes place in the dying animal tissue, making dextrose and maltose available. In the investigation here reported, tests were conducted to demonstrate the presence of reducing sugar in freshly shucked oysters. The fermenting or souring of oysters, therefore, is due to fermentation of the available dextrose and maltose rather than to direct decomposition of glycogen.

The results of this study indicate that the decomposition of shucked oysters in the beginning is probably due primarily to the activities of some members of the Serratia, Pseudomonas, Proteus, Clostridium, Bacillus, Aerobacter, and Escherichia groups of bacteria. Later in the course of the spoilage, streptococci, lactobacilli, and yeasts find more suitable conditions for development, until in the very late stages of decomposition the high-dilution plates made from the oysters, which become very sour and putrid, contain almost exclusively colonies of these three groups of organisms. In such oysters the hydrogen-ion concentration is between P_H 5.0 and 4.6.

Washing the shucked oysters in fresh water or in brine as conducted in these experiments had no apparent effect on the character of the bacterial flora causing spoilage. The oysters employed in these experiments were divided into two lots. The shells of the oysters in one lot were scrubbed thoroughly with a stiff brush under running water. The shells of the other lot were left unwashed. The oysters in both lots were then shucked in their own liquor. The oysters of one batch from washed shells and of one batch from unwashed shells were drained, washed in a colander in running fresh water for a few minutes, allowed to stand in a pan of fresh water for 10 minutes, drained, and stored. The remaining batch from each lot of shell oysters was drained, washed in a colander in running fresh water for a few minutes, allowed to stand in a pan of 2.5 per cent salt solution for 10 minutes, drained, and stored. The organisms which were found responsible for the decomposition were isolated consistently from the washed as well as the unwashed oysters. In commercial practice the use of a "blower" might, in some slight degree, affect the character of the flora, although various investigators have reported the impossibility of removing bacteria from the digestive canal of the oyster by any method of washing. Regardless of the effect of washing on the bacterial flora, good commercial practice calls for a thorough washing of shucked oysters in a weak brine to remove dirt, pieces of shell, seaweed, and other foreign material, and to produce as clean a product as possible.

SUMMARY

The decomposition of shucked oysters is apparently due primarily to the presence and development of certain members of the following groups of bacteria: Serratia (water and soil bacteria producing red pigment), Pseudomonas (the fluorescent group), Proteus, Clostridium (the anaerobic spore-forming group), Bacillus (the aerobic spore-forming group), Escherichia (the colon bacteria), Aerobacter (the aerogenes group), Streptococcus, and Lactobacillus. Some decomposition is also caused by certain yeasts.

The streptococci, lactobacilli, and yeasts predominate in the late stages of spoilage, when the hydrogen-ion concentration is between P_H 5.0 and P_H 4.6, while the other forms predominate in the early stages of decomposition.

The remaining organisms, comprising the microflora of oysters, are, for the most part, ordinary nonspore-forming soil and water bacteria and yeasts.

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THE EGG-PRODUCING CAPACITY OF ASCARIS LUMBRICOIDES[1]

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INTRODUCTION

In connection with the life history of *Ascaris lumbricoides*, concerning which there have been important developments in recent years as a result of the researches of Stewart and other investigators, the question of the capacity for egg production is of considerable interest. It is well known that this worm is very fertile, but a great deal of uncertainty exists as to the number of eggs that a female may produce, the various estimates in the literature ranging up to as many as 80,000,000 eggs. The writer has undertaken to make an approximate count of the number of eggs, fully developed and in process of development, in the female Ascaris. Various investigators (Cloquet,[2] Eschricht,[3] Siebold,[4] Leuckart,[5] and others) have described the reproductive system of the female *Ascaris lumbricoides*, with more or less completeness, and only a brief review of certain aspects of its anatomy, important in relation to the question of capacity for egg production, need here be given.

SEX ORGANS OF FEMALE ASCARID

The two uteri branch off from the vagina at its inner end about 6 mm. from the vulva. Bakker [6] concludes from the study of numerous specimens that as the body length becomes greater the uterine length does not increase proportionately. She found that whereas ascarids 19 and 20 cm. long contained uteri 17 and 18 cm. in length, respectively, ascarids 32 and 35 cm. long had uteri 22 and 24 cm. in length, respectively. The uteri lie in a fairly straight course backward from the genital pore, the distal portion of each turning forward. At this distal end is situated the receptaculum seminis, beyond which is the oviduct and then the long filiform ovary which coils back and forth in the body cavity to such an extent that a cross section of the body shows 20 or 30 sections of the genital tubes. Each ovary measures 120 to 200 cm. in length, five to eight times the total length of the worm itself.

A histological study of these various sex organs of the female ascarid shows the steps in the development of the ova. The extremely delicate free or distal portion of the ovary (about 225 μ in diameter) is the germinal zone; it contains a mass of protoplasm with an abundance of nuclei, or germinal vesicles, scattered through it. At a distance somewhat farther from the free end of the ovary, the protoplasm gradually forms around the vesicle and distinct cells or ova begin to appear (fig. 1). Then follows the developmental zone of the ovary, in which the ova have elongated and arranged themselves in wreaths around a rachis, an axial protoplasmic cord which supplies them with nutriment. The ova are from 100 to 200 μ in length, and as viewed in a longitudinal section of the ovary are 9 to 10 μ broad at the outer end and somewhat narrower at the inner end, which is attached to the rachis (fig. 2). In cross section of the ovary they are about 7μ broad at the outer end and sharply pointed at the attachment to the rachis (fig. 3). The germinal vesicle is situated near the broad end of the ovum in its early development, when the ovum has a length of 100 μ (fig. 4), but later it gradually approaches the center, at

[1] Received for publication August 22, 1924; issued July, 1925.

[2] CLOQUET, J. ANATOMIE DES VERS INTESTINAUX. 130 p., illus. Paris. 1824.

[3] ESCHRICHT, D. F. INQUIRIES EXPERIMENTAL AND PHILOSOPHICAL, CONCERNING THE ORIGIN OF INTESTINAL WORMS. Edinb. New Phil. Jour. 31: 314–356, illus. 1841.

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[6] BAKKER, C. R. OVER DE IDENTITEIT VAN ASCARIS LUMBRICOIDES EN ASCARIS SUILLA. Tijdschr. Vergelijk. Geneesk. 6: 160–230, illus. 1921.

which time the ovum is about 200 μ long (fig. 2).

The rachis disappears as the character of the ovary changes to form the oviduct, and the ova separate from each other and gradually assume a more or less oval form. They are fertilized by the sperm in the receptaculum seminis, and the shell is developed while they are in the posterior end of the uterus.

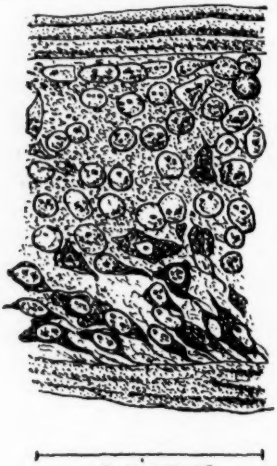

FIG. 1.—*Ascaris lumbricoides*. Germinal zone of ovary

METHOD OF COUNTING EGGS

The writer has attempted to count the eggs in two adult specimens of *Ascaris lumbricoides*. The genital system of the worm was carefully dissected, then floated in water while the many loops of tubules were disentangled, and then the uteri and ovaries were carefully measured. After that, portions were cut off at different levels, mounted in celloidin, and cross and longitudinal sections made of each portion of tubule. After staining and mounting the section, the eggs were counted in several sections from each of the selected portions, and the average computed. In the cross sections of ovary and uterus (figs. 3 and 5) the total number of eggs present was counted (see columns *a* and *a'* in the tables); in the longitudinal sections of ovary and uterus (figs. 2 and 6) a linear count was made of the eggs arranged consecutively along a known length of the wall of the tube, and from that length was computed the approximate number in a 1 mm. length (see columns *b* and *b'* of the tables). By multiplying the number of eggs in cross section by the linear count for

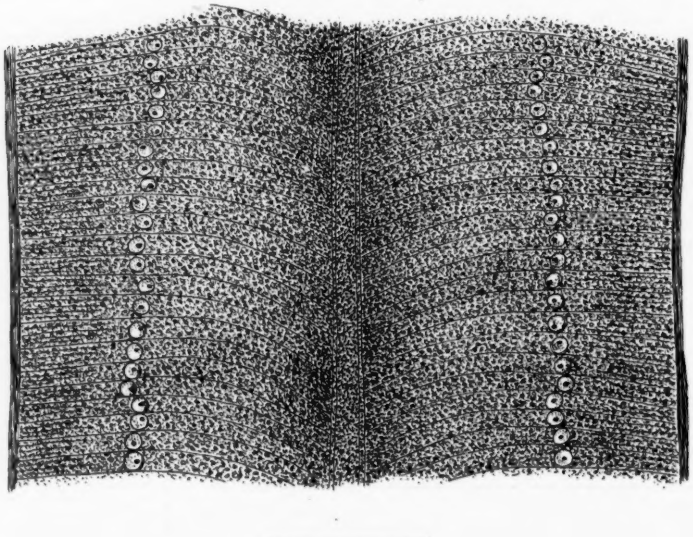

FIG. 2.—*Ascaris lumbricoides*. Longitudinal section of ovary

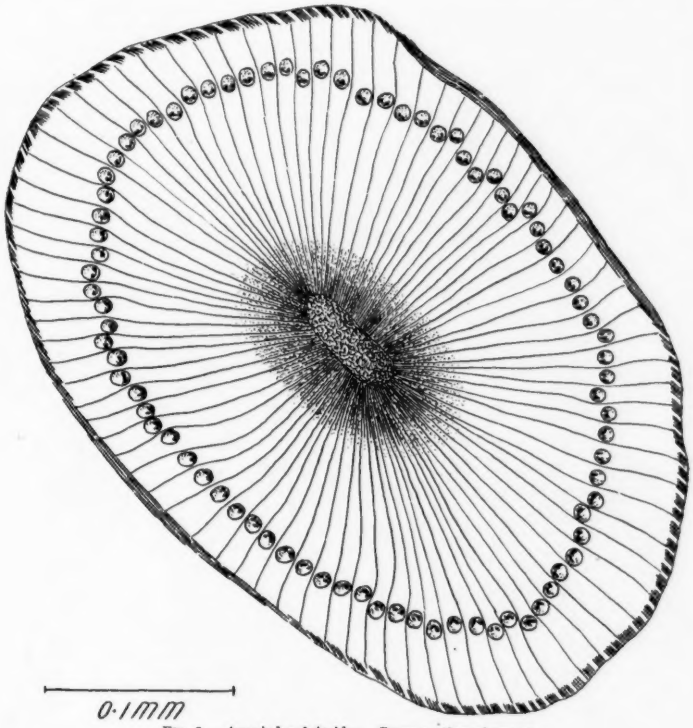

FIG. 3.—*Ascaris lumbricoides*. Cross section of ovary

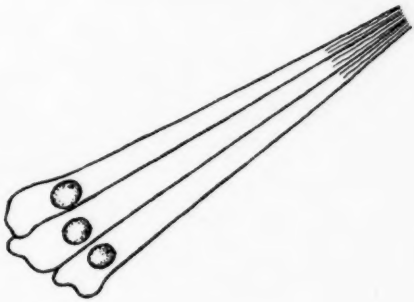

FIG. 4.—*Ascaris lumbricoides*. Ova from ovary

1 mm. longitudinal section, there was obtained the approximate total number of eggs in a 1 mm. length of the tube, either ovary or uterus (columns *c* and *c'* of the tables). Such counts were made of the first specimen (Table I, Ascaris A) on 5 portions of ovary (13 cross sections and 13 longitudinal sections being counted), and on 4 portions of uterus (12 cross sections and 12 longitudinal sections being counted). The average number of eggs in a 1 mm. length of ovary, as computed from the five parts selected, was 7,431.2; that in a 1 mm. length of uterus, as computed from the 4 parts selected, was 5,642.85. These numbers, multiplied by the total length of the ovaries and uteri, respectively, gave 23,668,372 as the total number of eggs in the ovaries, and 2,821,425 in the uteri, or a sum total of 26,489,797 eggs in that specimen of *Ascaris lumbricoides*.

The same procedure was followed with the second specimen, except that 18 cross and longitudinal sections of 6 portions of ovary and 9 cross and longitudinal sections of 3 portions of the uterus were counted (Table II, Ascaris B). The total number of eggs in the ovaries was 25,144,591 and that in the uteri 2,413,224, the total number in the worm being 27,557,815. For this second specimen a smaller worm than the first was deliberately chosen. The fact that the number of eggs in the

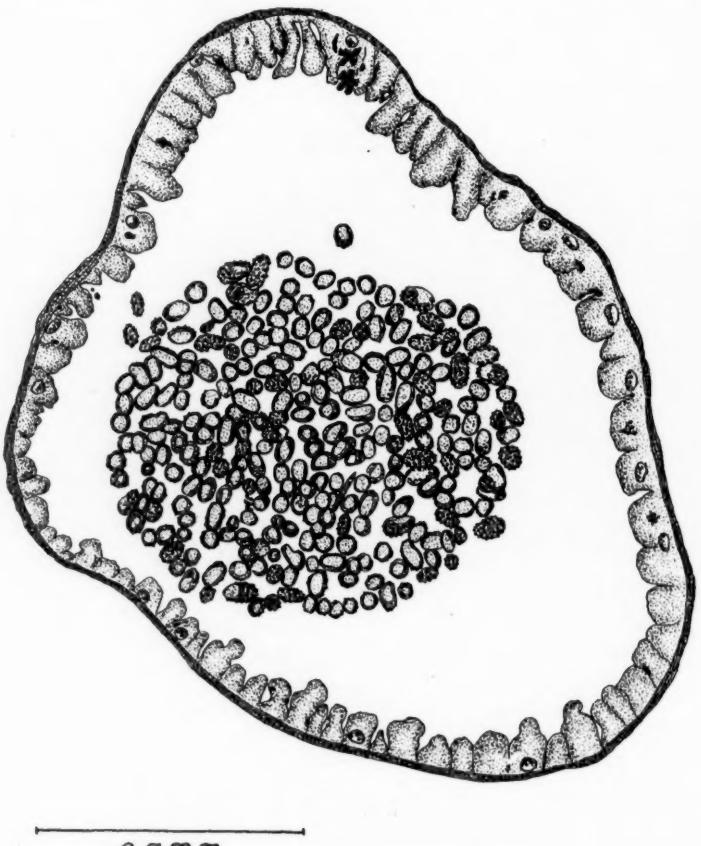

FIG. 5.—*Ascaris lumbricoides*. Cross section of uterus

ovaries, and especially in the germinal zone, of the smaller and therefore presumably younger female (Ascaris B) was greater than in the larger specimen (Ascaris A), while the number in the uteri of the younger worm was smaller and the total number of eggs in ovaries and uteri was slightly greater, is perhaps significant as indicating that in the older worm more eggs had matured and been deposited.

The free end of the ovary is but 1-25th of a line in diameter. A transversal section of the ovary shows the number of ova around the rachis to be about 50 and their diameter to be about 1-500th part of a line. Hence in the space of one line there will be 500 wreaths or stars of 50 eggs each, so affording 25,000 ova. The length of each horn of the female organ is about 16 ft. or 2,304 lines, which for the two horns gives 4,608 lines. If the ova, therefore, were of the same diameter throughout, their number would amount to 25,000×4,608, but as they augment in size as they proceed from the ovary to the uterus, till at last they attain a diameter of 1-60th of a line, they will not form more than 60 wreaths or 3,000 eggs in one line within the uterus. Thus, supposing the diameter of the eggs to increase proportionately throughout the length of the female organs, we may calculate the number of ova, on an average, at $\frac{25,000+3,000}{2}$, or 14,000 in each line, giving a total number of eggs at 14,000×4,608, of course more than 64,000,000.

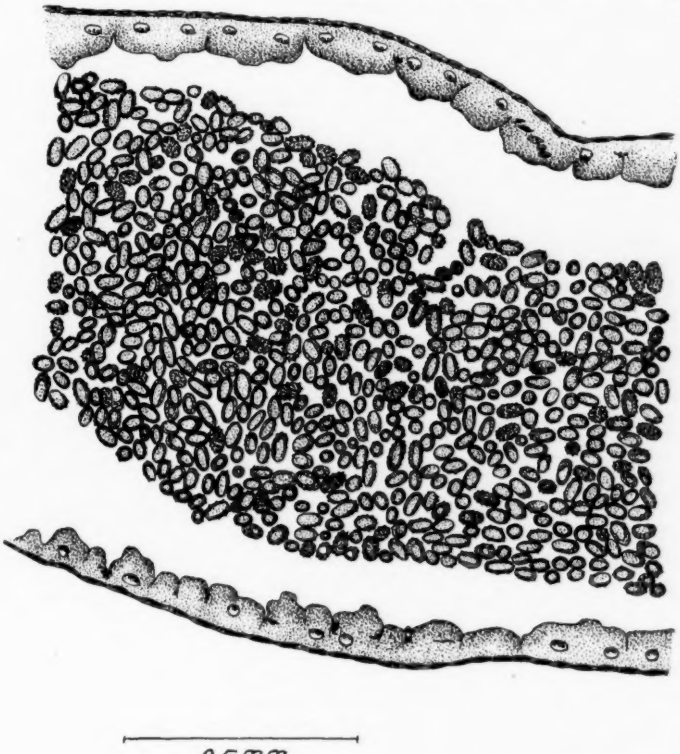

FIG. 6.—*Ascaris lumbricoides.* Longitudinal section of uterus

DISCUSSION OF RESULTS OF OTHER INVESTIGATORS

The figures obtained in these counts, 26,000,000 and 27,000,000 as the total number of eggs in a female ascarid, are considerably lower than those obtained by Eschricht (footnote 3) and by Leuckart (footnote 5), namely 64,000,000 and 70,000,000, respectively.

Eschricht's computations are as follows:

Eschricht evidently assumes in these computations that there are still 50 eggs in a cross section of the uterus as there were in the ovary, arranged around the rachis; this is indicated in his statement that the ova "will not

TABLE I.—*Ascaris* A. *Egg count in female 29 cm. long*

Ovaries (3,185 mm. combined length)			Uteri (500 mm. combined length)		
a In cross section	*b* Consecutive in 1 mm. longitudinal section	*c* Total in 1 mm. length of ovary ($a \times b$)	*a* In cross section	*b'* Consecutive in 1 mm. longitudinal section	*c'* Total in 1 mm. length of uterus ($a' \times b'$)
	Eggs	*Eggs*		*Eggs*	*Eggs*
I. Germinal zone:			I. 122	21.2	
35	144.9		114	17.7	
48	125.3		144	23.7	
Average 42	Average 135.1	5,674.2	Average 126.7	Average 20.9	2,648.0
II. 88	66.2		II. 571	27.3	
82	72.2		536	24.4	
91	72.2		545	23.6	
Average 87	Average 70.2	6,107.4	Average 550.7	Average 25.1	13,822.6
III. 75	65.8		III. 237	21.6	
78	82.3		221	21.3	
75	88.0		225	22.9	
Average 76	Average 78.7	5,981.2	Average 227.6	Average 21.9	4,984.4
IV. 76	111		IV. 45	24.7	
78	125		42	25.5	
Average 77	Average 118	9,086.0	46	25.3	
			Average 44.3	Average 25.2	1,116.4
V. 97	110.4		Average		5,642.85
93	102.6				
95	112.4				
Average 95	Average 108.5	10,307.5			
Average		7,431.2			

Total number of eggs in ovaries (7,431.2×3,185), 23,668,372.
Total number of eggs in uteri (5,642.85×500), 2,821,425.
Total number of eggs in the ascarid, 26,489,797.

TABLE II.—*Ascaris* B. *Egg count in female 27.5 cm. long*

Ovaries (3,115 mm. combined length)			Uteri (360 mm. combined length)		
a In cross section	*b* Consecutive in 1 mm. longitudinal section	*c* Total in 1 mm. length of ovary ($a \times b$)	*a'* In cross section	*b'* Consecutive in 1 mm. longitudinal section	*c'* Total in 1 mm. length of uterus ($a' \times b'$)
	Eggs	*Eggs*		*Eggs*	*Eggs*
I. Germinal zone:			I. 320	18.2	
148	123.4		376	19.8	
128	145.7		326	21.9	
185	127.6		Average 340.6	Average 19.9	6,777.9
Average 153.6	Average 132.2	20,305.9	II. 474	16.8	
II. 41	87.9		442	21.7	
41	86.0		462	22.1	
41	90.0		Average 459.3	Average 20.2	9,277.9
Average 41	Average 87.9	3,603.9	III. 306	16.8	
III. 56	69.2		218	15.9	
56	65.3		287	12.3	
60	72.2		Average 270.3	Average 15.0	4,054.5
Average 57.3	Average 68.9	3,948.0			
IV. 62	120.4		Average		6,703.4
62	108.7				
64	115.1				
Average 62.6	Average 114.7	7,180.2			
V. 67	115.6				
65	91.0				
68	110.1				
Average 66.6	Average 105.6	7,032.9			
VI. 54	110.8				
60	115.6				
56	110.8				
Average 56.6	Average 112.4	6,361.8			
Average		8,072.1			

Total number of eggs in ovaries (8,072.1 × 3115), 25,144,591.5.
Total number of eggs in uteri (6,703.4 × 360), 2,413,224.0.
Total number of eggs in the ascarid, 27,557,815.5.

form more than 60 *wreathes* in one line within the uterus." The eggs in the uterus are no longer the slender, elongate ova arranged radially around a rachis but are mature and unattached, and the counts in a cross section are seen in the present investigation to vary greatly, from 42 to 571 in such a section. Therefore, whereas Eschricht gives 3,000 eggs to a line, or, assuming that Eschricht's line was one-twelfth of a Paris inch of 27 mm. as used in Germany at the time his paper was published, 1,333 eggs to a 1 mm. length, in the uterus, the present writer found 5,642 and 6,703 to a 1 mm. length in the 2 specimens used.

The greatest difference, however, in the data given by Eschricht and those of this investigation is in the total lengths of the genital systems. Eschricht gives the length as 4,608 lines, which would be approximately 10,408 mm. The combined lengths of ovaries and uteri in the 2 specimens considered in this paper, however, were 3,684 mm. and 3,475 mm., respectively. In 3 other specimens measured by the writer the measurements were as follows: A female ascarid 28 cm. long had total uterine length of 360 mm. and total ovarian length of 3,520 mm.; in a specimen 28.5 cm. long the figures were 440 mm. and 3,650 mm., respectively; in a specimen 29 cm. long the figures were 460 mm. and 3,840 mm., respectively. Leuckart gives the length for a smaller worm (15.7 cm. in length) as 300 to 500 mm. for the combined length of uteri, and 2,500 for that of the ovaries. It seems then that Eschricht's measurements, two and one-half to three and one-half times as great as the above figures, are probably incorrect.

Leuckart computes the number of ova in an ascarid in the following manner. He states that the length of the ovary was 1,200 (presumably 1,200 mm.), the base of each cone-shaped ovum in it 0.04 mm., and the number of ova in a cross section 100. From these data he concludes that the total number of eggs in each ovary would be 30,000,000, or 60,000,000 for both. The uteri, he says, contain an additional 10,000,000 to 11,000,000 eggs. There is apparently some error in Leuckart's computations, as the above data would give 3,000,000 and 6,000,000 instead of 30,000,000 and 60,000,000. It is possible, however, that the discrepancy lies in the figure 0.04 mm. as the base of the ovum; none of the ovarian ova measured by the present writer exceeded 10 μ, the range being 6.5 to 10 μ, so that it is possible that Leuckart meant 4 μ instead of 40 μ, in which case his 30,000,000 and 60,000,000 result would be mathematically correct.

Another important difference between his counts and the present writer's is that for the cross section of the ovary Leuckart states that there were 100 or more ova around the rachis (although his figure shows only 48), whereas the counts in the present investigations varied considerably in different parts of the ovaries and in the two different specimens. In Ascaris A there was an average of 84 ova, and in Ascaris B an average of 57 in such sections. Leuckart does not seem to have taken into consideration the germinal zone of the ovary in which no rachis is present.

It is seen thus that both the computations of Eschricht and those of Leuckart are confused, apparently containing either incorrect observations or deductions or, as far as it is possible to judge from the data, being mathematically unsound, and their counts of 64,000,000 and 70,000,000, respectively, can not be accepted as completely trustworthy. It was because of this that the present investigation was undertaken and the counts made that are recorded herein.

SUMMARY

This study of American material of *Ascaris lumbricoides* indicates that the total number of eggs, fully developed and in process of development, in the female is from 26,000,000 to 27,000,000, and the former estimates of Eschricht and of Leuckart, 64,000,000 and 70,000,000, respectively, are apparently incorrect.

A STUDY OF ESSENTIAL PLANT FOODS RECOVERABLE FROM THE MANURE OF DAIRY COWS [1]

By C. F. WELLS and B. A. DUNBAR, *Chemistry Department, South Dakota State College*

INTRODUCTION

The problem briefly discussed in this article grew out of certain queries suggested by analytical results which the writers obtained during the progress of their work done in cooperation with the Dairy Department of South Dakota State College upon the determination of digestion coefficients which characterized dairy cattle of varied types under the feeding of soybean hay and alfalfa hay as variants.[2]

The work here reported offers a measure of direct information on the important subject of the degree of recovery of potash, phosphorus, and nitrogen from the manures of dairy cattle during lactation periods. These results also afford a general comparison between the quantities of potassium, phosphorus, and nitrogen recoverable in the manure and the amount of milk simultaneously produced by the same cattle. This work is by no means complete, but it is felt it is capable of expansion into broader research leading to more complete generalization than was possible with the limited number of subjects used in these digestion trials.

EXPERIMENTAL DATA

These digestion trials were conducted during two periods of five days each; the first in February and the second in April of 1924. For the trials four dairy cows, in lactation, were fed corn silage, oats, corn, and oil meal, as constants, in approximately the same quantities of each per day. The variant feeding material consisted of constant daily rations of soybean hay during the first trial and of alfalfa hay during the second. Each of these feeding materials, both constants and variables, was from the same lot originally purchased for the experiments, and therefore only one analytical problem was presented in case of each of them.

The cattle used as subjects were of the following breeds, and are hereinafter referred to by numbers as assigned here: Cow No. 1, Jersey; cow No. 2, Ayrshire; cow No. 3, Guernsey; cow No. 4, Holstein. All were of the ordinary farm grade of cows, of about the same age, ranging closely around 5 years. At the beginning of the trials all were apparently in perfect health, and they were considered to be in equally good condition at the close of the experiment. At all times during the trials, as well as during the several weeks of preliminary conditioning to accustom them to the constant feeds and the methods of manure collection, the cows were allowed free access to salt and water and were cleanly bedded and stalled in comfort. This careful attention was also given in the interim between the trials.

During the trials the feces and urine were carefully and thoroughly collected during 24-hour periods, and a composite sample of each collection was kept in air-tight glass containers. The five composites for each 5-day trial period were analyzed at the close of the period. The refuse feeding material was also collected at the close of the trial period and analyzed for the three plant-food constants, to be used as a drawback upon the amount of the several materials fed in obtaining actual consumption of feed per day. In computing the weights of each constant actually consumed per day, the totals for the trial periods were divided by five, and they are given on this basis in the tables. In the analyses of feeds and liquid and solid manures the writers, with but little deviation, followed recognized methods of analysis, and for their immediate purposes only nitrogen, potassium oxide, and phosphorus (as the pentoxide) were considered. One-hundred-gram samples were put into solution by the use of concentrated sulphuric acid of specific

[1] Received for publication Aug. 18, 1924; issued July, 1925.

[2] This work was in part fulfillment of requirements for the degree of Master of Science, and was expected to show the relative feeding values of the hays in question. The writers are therefore indebted to the dairy department for certain data from which, by further analytical investigation, the material for this paper has been obtained.

gravity 1.84 and under gentle heat. The original moist composites were used to obviate loss of nitrogen on drying and for comparison of the data with those on the urine composites. This preliminary solution was made up to volume with distilled water, and aliquots of the diluted solution were used for analysis. Potassium oxide was determined by the perchlorate method, phosphorus pentoxide by the volumetric molybdate method, and nitrogen by the Kjeldahl process. All results so obtained are reported in the following tables:

TABLE I.—*Analysis of feeds, original moist basis*

	K_2O	P_2O_5	N
	Per cent	*Per cent*	*Per cent*
Soybean hay	2.290	0.490	1.344
Alfalfa hay	2.260	.960	2.660
Corn silage	.300	.146	.388
Oats	.540	1.140	2.016
Corn	.407	.850	1.596
Oil meal	1.270	1.860	5.656

At the close of the first 5-day digestion trial, the refuse was carefully collected and weighed and a composite sample analyzed. This refuse was found to be almost wholly the stems of soybean hay. At the close of the second trial period there was practically no refuse obtainable from the stalls, so only one analysis is reported. Results are given in terms of average pounds per day.

TABLE II.—*Analysis of refuse feed, first digestion trial*

Cow No.	Refuse feed	K_2O		P_2O_5		N	
	Lbs.	*Lb.*	*P. ct.*	*Lb.*	*P. ct.*	*Lb.*	*P. ct.*
1	0.345	0.0030	0.90	0.0004	0.12	0.0022	0.64
2	.528	.0048	.90	.0006	.12	.0030	.58
3	.806	.0072	.90	.0010	.12	.0088	1.10
4	1.600	.0144	.90	.0018	.12	.0018	.12

Using the data in Tables I and II, the quantities of the several materials fed per day being known by reference to the feeding tables for the two trials, the data given in Tables III and IV are easily computed. These tables present the actual amounts of each of the three constants fed, wasted, and consumed during the two trials.

TABLE III.—*Computation of oxides of potassium, phosphorus and nitrogen in feed*

FIRST DIGESTION TRIAL

Feed	Cow No. 1, Jersey				Cow No. 2, Ayrshire				Cow No. 3, Guernsey				Cow No. 4, Holstein			
	Feed per day	K_2O per day	P_2O_5 per day	N per day	Feed per day	K_2O per day	P_2O_5 per day	N per day	Feed per day	K_2O per day	P_2O_5 per day	N per day	Feed per day	K_2O per day	P_2O_5 per day	N per day
	Lbs.	*Lb.*	*Lb.*	*Lb.*	*Lbs.*	*Lb.*	*Lb.*	*Lb.*	*Lbs.*	*Lb.*	*Lb.*	*Lb.*	*Lbs.*	*Lb.*	*Lb.*	*Lb.*
S. B. hay	14	0.3206	0.0686	0.1882	10	0.2290	0.0490	0.1344	10	0.2290	0.0490	0.1344	12.8	0.2930	0.0628	0.1720
Silage	30	.0900	.0438	.1164	25	.0750	.0364	.0970	25	.0750	.0364	.0970	30	.0900	.0438	.1164
Oats	4	.0218	.0456	.0806	4	.0218	.0456	.0806	4	.0218	.0456	.0806	4	.0218	.0456	.0806
Corn	5	.0204	.0424	.0798	5	.0204	.0424	.0798	4	.0162	.0340	.0638	5	.0204	.0424	.0798
Oil meal	1	.0126	.0186	.0566	1	.0126	.0186	.0566	1	.0126	.0186	.0566	1	.0126	.0186	.0566
Total		.4654	.2190	.5216		.3588	.1920	.4484		.3546	.1836	.4324		.4378	.2132	.5054
Less refuse		.0030	.0004	.0022		.0048	.0006	.0030		.0072	.0010	.0088		.0144	.0018	.0018
Total consumed		.4624	.2186	.5194		.3540	.1914	.4454		.3474	.1826	.4236		.4234	.2114	.5036

SECOND DIGESTION TRIAL

Alfalfa	14	0.3164	0.1344	0.3724	10	0.2260	0.0960	0.2660	10	0.2260	0.0960	0.2660	12	0.2712	0.1152	0.3192
Silage	30	.0900	.0438	.1164	25	.0750	.0364	.0970	25	.0750	.0364	.0970	30	.0900	.0438	.1164
Oats	4	.0218	.0456	.0806	4	.0218	.0456	.0806	4	.0218	.0456	.0806	4	.0218	.0456	.0806
Corn	5	.0204	.0424	.0798	5	.0204	.0424	.0798	4	.0162	.0340	.0638	5	.0204	.0424	.0798
Oil meal	1	.0126	.0186	.0566	1	.0126	.0186	.0566	1	.0126	.0186	.0566	1	.0126	.0186	.0566
Total		.4612	.2848	.7058		.3558	.2390	.5800		.3516	.2306	.5640		.4160	.2656	.6526

TABLE IV.—*Analysis of urine* [a] *(both digestion trials)*

Animal	First digestion trial			Second digestion trial		
	K_2O	P_2O_5	N	K_2O	P_2O_5	N
	Per cent	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*
Cow No. 1	1.050	0.003	0.370	1.470	0.004	1.147
Cow No. 2	1.460	.004	.663	1.310	.004	1.120
Cow No. 3	1.660	.005	.794	1.760	.006	1.532
Cow No. 4	1.490	.003	.734	1.530	.004	1.286

[a] During both periods the urine and feces were carefully and separately collected, and separate analyses were made of composite samples of each collection.

TABLE V.—*Analysis of feces* [a] *(both digestion trials)*

Animal	First digestion trial			Second digestion trial		
	K_2O	P_2O_5	N	K_2O	P_2O_5	N
	Per cent	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*
Cow No. 1	0.050	0.140	0.340	0.080	0.140	0.386
Cow No. 2	.050	.170	.318	.090	.160	.348
Cow No. 3	.050	.160	.312	.070	.160	.347
Cow No. 4	.070	.120	.280	.060	.130	.342

[a] During both periods the urine and feces were carefully and separately collected, and separate analyses were made of composite samples of each collection.

TABLE VI.—*The daily urine and feces, and the constants contained therein*

FIRST DIGESTION TRIAL

Animal	Urine per day	Feces per day	K_2O per day			P_2O_5 per day			N per day		
			In urine	In feces	Total	In urine	In feces	Total	In urine	In feces	Total
	Pounds	*Pounds*	*Pound*	*Pound*	*Pound*	*Pound*	*Pound*	*Pound*	*Pound*	*Pound*	*Pound*
Cow No. 1	23.90	67.59	0.2510	0.0338	0.2848	0.0008	0.0946	0.0954	0.0884	0.2298	0.3182
Cow No. 2	21.30	56.40	.3110	.0280	.3390	.0008	.0958	.0966	.1412	.1794	.3206
Cow No. 3	14.45	56.48	.2398	.0282	.2680	.0008	.0902	.0910	.1148	.1774	.2922
Cow No. 4	12.85	77.75	.1914	.0547	.2461	.0004	.0932	.0936	.0944	.2174	.3118

SECOND DIGESTION TRIAL

Cow No. 1	22.40	65.50	0.3292	0.0524	0.3816	0.0010	0.0916	0.0926	0.2568	.2528	0.5096
Cow No. 2	21.95	52.90	.2874	.0476	.3350	.0008	.0846	.0854	.2458	.1840	.4298
Cow No. 3	14.90	46.80	.2622	.0328	.2950	.0008	.0748	.0756	.2282	.1624	.3906
Cow No. 4	19.00	58.80	.2906	.0352	.3258	.0008	.0764	.0772	.2444	.2010	.4454

Table VII, based on all the data in the preceding tables, is given to facilitate a comparison between the daily milk production and the quantities of potash, phosphorus, and nitrogen recovered daily in the liquid and solid manures.

TABLE VII.—*Daily milk production, and pounds of potash, phosphorus, and nitrogen recovered*

FIRST DIGESTION TRIAL

Animal	Milk produced	K_2O			P_2O_5			N		
		Consumed	Voided	Recovered	Consumed	Voided	Recovered	Consumed	Voided	Recovered
	Pounds	*Pound*	*Pound*	*Per cent*	*Pound*	*Pound*	*Per cent*	*Pound*	*Pound*	*Per cent*
Cow No. 1	24.16	0.4624	0.2848	61.59	0.2186	0.0954	43.64	0.5194	0.3182	61.26
Cow No. 2	22.66	.3540	.3390	95.76	.1914	.0966	50.46	.4454	.3206	71.98
Cow No. 3	18.44	.3474	.2680	77.14	.1826	.0910	49.83	.4236	.2922	68.98
Cow No. 4	25.42	.4234	.2458	58.05	.2114	.0936	44.27	.5036	.3118	61.91
SECOND DIGESTION TRIAL										
Cow No. 1	20.74	0.4612	0.3816	82.74	0.2848	0.0926	32.51	0.7058	0.5096	72.20
Cow No. 2	23.36	.3558	.3350	94.15	.2390	.0854	35.73	.5800	.4298	74.10
Cow No. 3	18.92	.3516	.2950	83.91	.2306	.0756	32.78	.5640	.3906	69.25
Cow No. 4	23.58	.4160	.3258	78.31	.2656	.0772	29.06	.6526	.4454	68.25

CONCLUSION

During the first of the two trials the amount of potash recovered in the manure was between 58 and 96 per cent of the total fed; the recovery of phosphates ranged between 44 and 50 per cent of the total fed; and the recovery of nitrogen ranged from 61 to 72 per cent. In the second trial the ranges were, for potash, 78 to 94 per cent; for phosphates, 29 to 36 per cent; for nitrogen, 68 to 74 per cent. From these results it seems fair to presume that the dairyman may expect to recover to his land approximately four-fifths of the potash, two-fifths of the phosphates, and two-thirds of the nitrogen fed to lactating cows. This is a saving of feed values that is by no means insignificant.

Contrary to such information as the writers have been able to gain from the literature, they have found that, after taking all precautions for accuracy of analysis, blanks having been run on the reagents used, there is an appreciable and measurable quantity of phosphorus voided in the urine.

SUMMARY

Recognizing that the drawing of general conclusions on the basis of these limited data may not be warranted, the writers submit certain inferences which are apparently warranted; but these inferences are, of course, subject to amendment by further investigation and study.

The quantity of the different constants—nitrogen, phosphorus, and potassium—here examined varies quite markedly. There is consistently more of the potash and nitrogen recoverable in feces and urine than of phosphorus. This fact, however, by no means new to the literature, is but merely confirmatory.

The quantities of the nitrogen, phosphorus, and potassium vary strikingly with the type of animal. In this experiment the highest values returned to the soil through the voided material were returned by the Ayrshire, which also maintained a high milk production at the same time.

The quantities varied with the daily milk production. In a very general way, the recovery of potash and nitrogen was in inverse relation to milk production, while the phosphorus recovery paralleled the production of milk. But the last-named constant is seen to be less influenced by the amount of milk produced than the other two.

The above-suggested relation between milk production and the potash and nitrogen voided is more apparent in the instances wherein the daily milk production varies widely.

PHYSIOLOGICAL AND BIOCHEMICAL STUDIES ON CEREALS. IV. ON THE PRESENCE OF AMINO ACIDS AND POLYPEPTIDES IN THE UNGERMINATED RYE KERNEL [1]

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INTRODUCTION

In continuing the work reported in previous papers on wheat (*Triticum vulgare*) (*10*),[2] oats (*Avena sativa*) (*11*), and corn (*Zea mais*) (*12*), it was found further that the ungerminated kernel of rye (*Secale cereale*) also contains polypeptides and amino acids. A careful perusal of the literature reveals no report of the presence of these compounds in the ungerminated rye kernel.

Einhof (*3*) was the first to report the presence in rye seed of albumin and of a protein soluble in alcohol. The occurrence of an alcohol-soluble protein was also reported by Heldt (*5*), who gave a complete analysis of it. According to Von Bibra (*1*), the albumin, "casein," and the alcohol-soluble protein in the rye kernel are identical with those in the wheat kernel, while according to Ritthausen (*17*) the rye kernel contains three proteins, namely, albumin, an alcohol-soluble protein (mucedin), and one soluble in dilute potassium hydroxide (gluten-casein).

From the work of Osborne (*13*), we now know definitely that the rye kernel contains five proteins, in the following proportions:

	Per cent
Albumin (leucosin)	0.43
Globulin (edestin) and proteose	1.76
Gliadin	4.00
Glutelin	2.44
Total	8.63

According to this investigator (*14*, *15*, *p. 79*, and *16*), wheat and rye yield similar quantities of albumins and globulins, which seem to be identical, and also the same quantity of gliadin.

Of the nonproteins, Schulze (*18*) reports that the seed of rye contains on the average 0.195 per cent, calculated on the basis of the oven-dried seed, or 9.4 per cent, calculated on its total nitrogen. Schulze concludes from his observations that these figures are more or less fluctuating, depending on the state of ripeness of the seed, the percentage of nonproteins being ordinarily higher in unripe seed. According to Schulze (*19*), there occurs in the seed of rye a nitrogenous phosphatide which contains about 2 per cent of phosphorus, while according to Czapek (*2*, *p. 157*), the seed contains 0.57 per cent of lecithin when calculated to its oven-dried state. As mentioned above, the presence of polypeptides and amino acids in the ungerminated rye kernel is shown for the first time in this paper, so far as the writers are aware.

EXPERIMENTAL DATA

For this work North Dakota No. 959, Reg. Rosen, and Von Rümker varieties of rye were used. The samples were first dried in an electric oven at about 50° C. for one to two days, then ground in a buhr mill, and passed through a 60-mesh sieve.

METHODS USED

The total and the protein nitrogen were determined according to Kjeldahl's and Stutzer's methods, respectively. The amino and the peptide nitrogen were estimated by the formol-titration method (*6*, *20*) as used by the senior author and described in previous publications (*7*, *8*, *9*, *10*, *11*, *12*). Some details of these and other methods will be described subsequently. General information concerning the chemical composition of the three varieties of rye will be found in Table I.

In Table I it will be noticed that the percentage of total nitrogen is highest in North Dakota No. 959, lowest in Reg. Rosen, while the figure for Von Rümker is between the two. The same relationship holds good for the non-

[1] Received for publication Aug. 6, 1924; issued July, 1925.
[2] Reference is made by number (italic) to "Literature cited," p. 992.

protein nitrogen and for the protein nitrogen when the latter is referred to the oven-dried substance. The reverse, however, is true when the protein nitrogen is referred to the total nitrogen. As to the ash content, Von Rümker rye is characterized by the highest percentage of ash, followed by Reg. Rosen and North Dakota No. 959 in the order named.

Inasmuch as both aqueous and alcoholic extracts were used for this work, we have determined the proportion of nitrogen that could be extracted by both solvents under certain conditions.

Ordinarily 25-gram portions of flour were treated with 500 c. c. of boiling-hot ammonia-free water and kept on the water bath for 30 minutes and then filtered or centrifuged. The solid resi-

TABLE I.—*Proportion of ash and of total, of protein, and of nonprotein nitrogen in the ungerminated rye kernel*

Variety of rye	Where and when grown	Total nitrogen, oven-dried rye	Protein nitrogen		Nonprotein nitrogen		Ash oven-dried rye
			Oven-dried rye	Total nitrogen	Oven-dried rye	Total nitrogen	
		Per cent	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*
North Dakota No. 959 rye.	Grown at Dickinson, N. Dak., in 1922.	2.35	2.01	83.40	0.40	16.60	1.79
		2.41	1.99	82.77	.42	17.23	1.79
		2.42	2.06	85.50	.35	14.50	1.78
		2.45	------	------	------	------	1.80
		2.43	------	------	------	------	1.78
		2.43	------	------	------	------	------
		2.41	------	------	------	------	------
Average		2.41	2.02	83.89	.39	16.11	1.79
Von Rümker rye, C. I. 133.	Grown at —— in 1923.	1.86	1.60	87.02	.24	12.98	2.13
		1.83	1.55	84.38	.29	15.62	2.13
		1.85	1.58	85.79	.26	14.21	2.06
		1.83	------	------	------	------	2.10
Average		1.84	1.58	85.73	.26	14.27	2.11
Reg. Rosen rye No. R 22, 198.	Grown at Parma, Mich., in 1922.	1.65	1.48	89.71	.17	10.29	2.04
		1.62	1.53	92.73	.12	7.27	2.02
		1.64	1.52	92.12	.13	7.88	2.04
		1.66	------	------	------	------	2.04
		1.67	------	------	------	------	------
		1.65	------	------	------	------	------
		1.67	------	------	------	------	------
Average		1.65	1.51	91.52	.14	8.48	2.04

TABLE II.—*Proportion of nitrogen extracted by water and alcohol*

Variety of rye	Nitrogen extracted by water		Nitrogen extracted by 92 per cent alcohol	
	Milligrams	In per cent of oven-dried flour	Milligrams	In per cent of oven-dried flour
North Dakota No. 959 Rye	50.9684	0.55	56.4043	0.152
	50.9123	.55	56.4183	.152
Average	------	.55	------	.152
Von Rümker Rye, C. I. 133	44.9581	.49	58.2395	.158
	45.8267	.50	58.9121	.160
Average	------	.50	------	.159
Reg. Rosen Rye No. R 22198	44.6499	.48	56.2782	.152
	43.8083	.47	55.7178	.150
Average	------	.48	------	.151

dues were treated once more in the same manner. The filtrates were then made up to 1 liter, of which 400 c. c. portions were oxidized according to Kjeldahl's method. In the case of alcohol, 100-gram portions of flour were extracted with boiling 92 per cent alcohol, the extracts being separated by suction on Buchner funnels from the solid residues, which were extracted once more in like manner. Filtrates and washings were now made up to 1,000 c. c., in 400 c. c. portions, from which the alcohol was first removed by distillation and the nitrogen then estimated in the residue according to the Kjeldahl method. The results are recorded in Table II.

Reference to Table II shows that both the aqueous and alcoholic extractions were quite uniform, but that water extracted about three times as much nitrogen as did the alcohol. This is perhaps due to a greater proportion of protein matter taken up by the water.

In order to demonstrate the presence of amino acids and polypeptides in the rye kernel a series of flasks containing definite quantities of flour were treated with boiling-hot ammonia-free water, which was followed by digestion on the steam bath for 15 to 30 minutes. The extract was now separated by filtration or centrifugalization from the solid residue, which was treated once more in like manner. The extracts were then concentrated under reduced pressure, the precipitates formed (proteins, bran, etc.) removed by centrifugalization, the supernatant liquid evaporated to dryness, extracted with 85 per cent alcohol, filtered, the alcohol distilled off, and finally evaporated to dryness. The dry residue, a dry yellow sirup, was taken up with hot water, filtered, cooled, and made up to 100 c. c. (or its multiple), of which two portions of 10 c. c. each were oxidized according to Kjeldahl's method. To the remaining solution sulphuric acid was added to a concentration of 5 per cent, which was followed by treatment with a phosphotungstic acid solution containing 5 grams of sulphuric acid and 20 grams of phosphotungstic acid per 100 c. c., using but a slight excess of the precipitant. After 24 hours the heavy precipitate was filtered out and thoroughly washed with a solution containing 5 gm. of sulphuric acid and 2.5 gm. of phosphotungstic acid per 100 c. c. The precipitate formed by phosphotungstic acid ordinarily removes any proteins, proteoses, and peptones present. That it also contained diamino acids was shown in the following manner: The precipitate was treated with barium hydroxide, the excess of which was removed with carbon dioxide, the whole was filtered off with suction, and the remaining cake thoroughly washed with hot water. Filtrate and washings were evaporated in vacuo practically to dryness, taken up with a few cubic centimeters of water, and filtered. The filtrate, which had a light-yellow color, gave the following reactions:

(1) Phosphotungstic acid gave at once a heavy white precipitate.
(2) Phosphomolybdic acid gave immediately a yellow precipitate.
(3) Silver nitrate gave a grayish precipitate soluble in excess of ammonia.
(4) Addition of neutralized formaldehyde caused the solution to become acid.
(5) Mercuric chloride gave a grayish flocculent precipitate.

The filtrate from the phosphotungstic precipitate was freed from sulphuric and phosphotungstic acids by treatment with calcium hydroxide to slight acidity, then with barium hydroxide to alkalinity, the excess of barium hydroxide being removed with carbon dioxide. The whole was now brought to a boil and filtered, the solid residue being extracted once more with hot ammonia-free water. The filtrate and washings were then concentrated under reduced pressure and made up to 100 c. c., of which two portions of 20 c. c. each were oxidized by the Kjeldahl method, while 50 c. c. were (on being freed from carbon dioxide, phosphoric acid, and coloring matter) formol-titrated, which gave the nitrogen of monoamino acids.

The presence of polypeptides was demonstrated as follows: The flour extract and the phosphotungstic acid precipitate were obtained exactly in the manner just described. Equally, the filtrate from the phosphotungstic precipitate was freed from sulfuric and phosphotungstic acids and finally concentrated in vacuo to 100 c. c., as outlined above. In two 10 c. c. portions the nitrogen was estimated according to the Kjedahl method. To the remaining 80 c. c. hydrochloric acid was added to a concentration of 20 per cent and hydrolyzed under a reflux condenser for 12 hours, in accordance with the observations of Fischer (*4*). The hydrolysate was now evaporated on the water bath to dryness, taken up with hot water, and distilled with magnesium oxide in order to remove the ammonia. The residue was then thoroughly extracted with hot water (which was previously freed from ammonia by long boiling), evaporated, and made up to 100 c. c., of which two portions of 20 c. c. each

were oxidized according to Kjeldahl's method, while 50 c. c. of the remaining solution were used for formol-titration.

For the estimation of the nitrogen of acid amides the extraction of the flour as well as the precipitation with phosphotungstic acid, etc., were effected as outlined in the case of the polypeptides, but the solution to which hydrochloric acid had been added to a concentration of 20 per cent was hydrolyzed for only 30 minutes. It was now evaporated to dryness, and the residue, which was taken up with hot water, was distilled with cream of magnesia, the ammonia being received in an Erlenmeyer flask containing N/10 H_2SO_4. The results are summarized in Table III.

Examination of Table III shows that when the results are referred to the water-soluble nitrogen the monoamino nitrogen is highest in Reg. Rosen (20.62 per cent), slightly lower in Von Rümker (20.10 per cent), and considerably lower in North Dakota No. 959 (13.56 per cent). As to the peptide nitrogen, it is highest in Von Rümker (30.96 per cent), somewhat lower in North Dakota No. 959 (29.36 per cent), and lowest in Reg. Rosen (14.55 per cent). Concerning the nitrogen of acid amides, it is highest in Von Rümker (18.50 per cent), lowest in Reg. Rosen (14.28 per cent), while the figure for North Dakota No. 959 is between the two. The outstanding feature of the results presented in Table III is the fact that the three varieties investigated contain not inconsiderable quantities of amino acids and polypeptides in their ungerminated kernels.

TABLE III.—*Distribution of the nonprotein nitrogen in the ungerminated rye kernel*

Variety of rye	Nitrogen of acid amids	Nitrogen of amino acids	Peptide nitrogen
Percentage of the water-soluble nitrogen of the rye kernel:	*Per cent*	*Per cent*	*Per cent*
North Dakota No. 959 Rye		13.96	29.53
		13.16	29.19
Average	16.32	13.56	29.36
Von Rümker Rye C. I. 133		19.71	30.64
		20.49	31.27
Average	18.50	20.10	30.96
Reg. Rosen Rye No. R 22198		20.77	14.80
		20.47	14.29
Average	14.28	20.62	14.55
Percentage of the total nitrogen of the rye kernel:			
North Dakota No. 959 Rye		3.18	6.73
		3.00	6.66
Average	3.72	3.09	6.69
Von Rümker Rye C. I. 133		5.28	8.21
		5.49	8.38
Average	4.96	5.39	8.30
Reg. Rosen Rye No. R 22198		6.01	4.29
		5.93	4.14
Average	4.14	5.97	4.21
Percentage of the oven-dried rye kernel:			
North Dakota No. 959 Rye		.077	.162
		.072	.161
Average	.090	.075	.162
Von Rümker Rye C. I. 133		.099	.153
		.103	.156
Average	.093	.101	.155
Reg. Rosen Rye No. R 22198		.100	.071
		.098	.069
Average	.069	.099	.070

It seemed of certain interest to extract the amino acids of the flour samples *directly* with alcohol instead of indirectly, as was done in the experiments described above. Hence definite quantities of flour were extracted with boiling 92 per cent alcohol, the extracts being separated by suction on a Büchner funnel from the residues, which were once more extracted in the same manner. The alcohol was removed from the combined extracts by distillation in a vacuum. The residues were alternately treated with ether (to remove fat) and hot water, and the ethereal and aqueous extracts separated in a separatory funnel. The aqueous extract was treated several times with small quantities of ether, while the ethereal extract was treated repeatedly with small quantities of water. The combined aqueous extracts, which contained the amino acids, were kept on the water bath for some time in order to remove some ether present, whereupon they were filtered. The solution was now slightly acidified with acetic acid and treated with 10 per cent tannic acid. The precipitate formed (chiefly protein) was filtered off, the filtrate was concentrated and made up to a definite volume, which was then treated with phosphotungstic acid in the manner already described. The filtrate from the phosphotungstic-acid precipitates was then treated with calcium hydroxide to slight acidity, then with barium hydroxide to alkalinity, saturated with carbon dioxide, boiled, filtered, the filtrate being concentrated under diminished pressure to 100 c. c. in two 20 c. c. portions, of which nitrogen was estimated according to Kjeldahl's method, while 50 c. c. were used for formol-titration. In this manner it was found that North Dakota No. 959 contains 19.95 per cent, 20.90 per cent, on the average 20.43 per cent, of monoamino nitrogen; while Reg. Rosen contains 35.51 per cent, 34.63 per cent, on the average 35.07 per cent, of monoamino nitrogen, when calculated on the basis of the *alcohol-soluble* nitrogen. These results, while not strictly comparable with those obtained with the aqueous extract, confirm in a general way the conclusion drawn from Table III that the ungerminated kernels of the rye varieties contain appreciable quantities of free amino acids.

TABLE IV.—*Distribution of the nonprotein nitrogen in the ungerminated kernel of wheat, oats, and corn*

Variety of cereal	Nitrogen of acid amides	Nitrogen of amino acids	Peptide nitrogen
Percentage of oven-dried *wheat* kernel:	*Per cent*	*Per cent*	*Per cent*
Fultz	0.026	0.032	0.084
Kanred	.053	.066	.111
Kubanka	.052	.041	.155
Marquis	.058	.054	.151
Percentage of total nitrogen of *wheat* kernel:			
Fultz	1.46	1.77	4.67
Kanred	1.88	2.34	3.89
Kubanka	1.72	1.35	3.13
Marquis	1.91	1.77	4.98
Percentage of water-soluble nitrogen of *wheat* kernel:			
Fultz	8.76	10.66	28.09
Kanred	12.99	16.25	26.86
Kubanka	12.61	9.91	37.76
Marquis	12.33	11.46	32.20
Percentage of oven-dried *oat* kernel:			
Swedish Select	.051	.064	.106
Victory	.029	.040	.073
Iowar	.046	.057	.084
Winter Turf	.027	.025	.032
Percentage of total nitrogen of *oat* kernel:			
Swedish Select	1.95	2.48	4.10
Victory	1.45	2.02	3.63

Variety of cereal	Nitrogen of acid amides	Nitrogen of amino acids	Peptide nitrogen
Percentage of total nitrogen of *oat* kernel—Continued.	*Per cent*	*Per cent*	*Per cent*
Iowar	1.93	2.35	3.49
Winter Turf	1.80	1.65	2.15
Percentage of water-soluble nitrogen of *oat* kernel:			
Swedish Select	13.69	17.39	28.71
Victory	10.86	15.08	27.16
Iowar	12.18	14.87	22.03
Winter Turf	15.01	13.79	17.88
Percentage of oven-dried *maize* kernel:			
Four County Corn	.032	.045	.069
U. S. Selection No. 77	.019	.040	.050
Hall Gold Nugget Selection No. 193	.021	.051	.036
Percentage of total nitrogen of *maize* kernel:			
Four County Corn	1.88	2.65	4.06
U. S. Sleection No. 77	1.19	2.52	3.14
Hall Gold Nugget Selection No. 193	1.44	3.49	2.47
Percentage of water-soluble nitrogen of *maize* kernel:			
Four County Corn	15.61	21.97	34.07
U. S. Selection No. 77	12.18	26.67	32.79
Hall Gold Nugget Selection No. 193	11.20	26.95	19.17

For convenient comparison the results obtained with the various cereals are presented in Table IV.

Although the figures in Tables III and IV are somewhat fluctuating, they indicate that the nitrogen percentage of amino acids is highest in the kernel of corn, lowest in that of wheat, and between the two for the rye and oat kernel. As to the percentage of peptide nitrogen, it is highest in the wheat kernel, followed by the kernels of maize, rye, and oats in the order named. The nitrogen percentage of acid amides may be said to be highest in the rye kernel and lowest in the wheat kernel, while the nitrogen percentage of the oat and maize kernel is between the two.

It may not be amiss here to mention that wheat and rye, each of which contains five proteins, namely, albumin, globulin, proteose, glutenin, and gliadin, thus showing a striking similarity, also display some similarity with regard to their nonproteins.

SUMMARY

Polypeptides and amino acids have been shown in this paper to occur in the ungerminated rye kernel.

The proportions of amino nitrogen in the varieties North Dakota No. 959, Von Rümker, and Reg. Rosen have been found to be, respectively, 3.09, 5.39, and 5.97 per cent, calculated on the basis of the total nitrogen; and 0.075, 0.101, and 0.099 per cent, calculated on the basis of the oven-dried kernel.

The percentages of peptide nitrogen are 6.69 for North Dakota No. 959, 8.30 for Von Rümker, and 4.21 for Reg. Rosen, calculated on the total nitrogen; and 0.162 for North Dakota No. 959, 0.155 for Von Rümker, and 0.070 for Reg. Rosen, calculated on the oven-dried kernel.

The nitrogen of acid amides in the varieties North Dakota No. 959, Von Rümker, and Reg. Rosen makes up, respectively, 3.72, 4.96, and 4.14 per cent, calculated to the total nitrogen; and 0.090, 0.093, and 0.069 per cent, calculated to the oven-dried kernel.

Inasmuch as we have shown that the ungerminated kernel of wheat, oats, maize, and rye contains polypeptides and amino acids, it seems safe to state that ungerminated kernels of the other cereals, such as rice, sorghum etc., also undoubtedly contain these substances.

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